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THE UNIVERSITY OF ALBERTA

ETHYLENE AND THE CHLOROPHYLL CONTENT OF DETACHED SPINACH LEAVES

by

© HUA HSU

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ETHYLENE AND THE CHLOROPHYLL CONTENT OF DETACHED SPINACH LEAVES submitted by HUA HSU in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Physiology and Biochemistry.

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ABSTRACT

This research was undertaken to study the effects of ethylene on the degradation of chlorophyll in spinach leaves. The influence of ethylene on chlorophyllase (chlorophyll chlorophyllidohydrolase, EC 3.1.1.14) activity and on the ultrastructure of the chloroplast, photosynthesis, respiration and changes in levels of protein was studied. Investigations were also made of the protective effect of CO_2, cytokinins and some inhibitors of protein synthesis (cycloheximide, chloramphenicol) against the action of ethylene.

In open systems, chlorophyllase activity was higher in ethylene-treated samples than in air-treated ones. The increase in chlorophyllase activity could be responsible for the greater decrease in chlorophyll in ethylene-treated samples. When spinach leaves were treated with ethylene in a sealed jar, the chlorophyll content of these leaves remained higher than that in open system probably because of reduced chlorophyllase activity in the presence of accumulated CO_2 inside the jar. Indeed, 1.2% CO_2 in a continuous stream of 50 ppm ethylene in air showed similar results.

Ethylene increased the soluble protein content of spinach leaves for a period of up to one day. The addition of cycloheximide (12 µg/ml) arrested the ethylene-induced chlorophyll degradation and chloramphenicol had only a slight effect. Thus, *de novo* synthesis of proteins was probably involved during ethylene-induced chlorophyll degradation.

The destructive effect of ethylene (50 ppm ethylene alone) on chlorophyll content was shown to be very high in the absence of CO_2.

At 10% CO_2 it acted rather synergistically with ethylene. However, at concentrations of 1%, 5%, 25% and 50% CO_2 carbon dioxide seemed to protect the degradation of chlorophyll by ethylene (50 ppm). The effect of CO_2 against ethylene-induced chlorophyll degradation was not directly proportional to its concentration.

Light intensity was also found to affect the chlorophyll content of spinach leaves treated with 50 ppm ethylene. The higher the light intensity, the lower was the chlorophyll content.

There was a quantitative difference in the pigments between ethylene-treated and air-treated spinach leaves as evidenced by Sephadex LH20 gel column, and thin layer chromatographic separation of chloroplast pigments. The decrease of carotenoids is contrary to what was expected in view of the increase of yellow pigmentation. Ethylene might have induced the degradation of chlorophyll, thus removing or unmasking the yellow color of carotenoids.

Antisenescent agents such as N-6-benzyladenine and kinetin were tested for their effect against the enhanced bleaching of chlorophyll in the presence of ethylene. These chemicals reduced the ethylene-enhanced bleaching. Kinetin was more effective than N-6-benzyladenine in intact spinach leaves and in disks.

Ethylene exerted no observable effect on the Hill reaction of chloroplasts isolated from spinach leaves whether these were concurrently or previously treated with 50 ppm ethylene for one day. However, ethylene did inhibit the Hill reaction of isolated chloroplasts from spinach leaves previously treated with 50 ppm ethylene for two or three days. Ethylene also reduced net CO_2 assimilation of spinach leaves. The Hill reaction of leaves treated with ethylene decreased by 40% as

compared to that of air controls at Day 2 and Day 3 of treatment, whereas the inhibition of net CO_2 assimilation was 60%.

The treatment of 50 ppm ethylene on spinach leaves for ultrastructure studies was for periods up to three days at light intensities of 50 and 2500 ft candles. The effects of ethylene on chloroplast ultrastructure were: swelling and discontinuity of thylakoids, dislocation of grana and sometimes invaginations of the inner membrane of the envelope and finally disappearance of thylakoid membranes. The disorganization of the thylakoid membrane systems increased with time and with higher light intensity. The osmiophilic globules usually occurred within the disorganized thylakoid area or at the end of the thylakoid and broke the continuity of the membrane system. These electron-dense bodies increased in number and size with increasing length of ethylene treatment.

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LIST OF ABBREVIATIONS

ACS	:	American Chemical Society
BSA	:	bovine serum albumin
Chl	:	chlorophyll
DNA	:	deoxyribonucleic acid
dpm	:	disintegrations per minute
h	:	hour
HEPES	:	N-2-hydroxyethylpeperazine-N-2-ethane-sulfonic acid
MES	:	2-(N-morpholino)-ethane-sulfonic acid
NADP	:	nicotinamide adenine dinucleotide phosphate
NBA	:	N-6-benzyladenine
ppm	:	parts per million
PS	:	photosystem
TLC	:	thin layer chromatography
TRICINE	:	N-tris(hydroxymethyl)methyl glycine
wt	:	weight

INTRODUCTION

A. Role of Ethylene in Leaf Senescence

1. Leaf senescence

Ethylene has been recognized as the natural triggering agent for senescence in many plant tissues (Cousins, 1910; Doubt, 1917; Denny, 1927; Corcker, 1932; Gane, 1937; Kidd and West, 1938; Hansen, 1943), including fruits and leaves. Osborne (1968) and Jackson and Osborne (1970) have reported from their studies of the pattern of ethylene production by *Phaseolus vulgaris* leaves that ethylene production increased as leaves aged and may be responsible for triggering senescence.

The degradation of chlorophylls of some fruits and other plant tissues has been shown to be accelerated by ethylene. Ripening of fruits can be manipulated by controlling the levels of available ethylene. This aspect has been successfully exploited for their commercial storage (see Ryall and Lipton, 1972).

In addition to controlling fruit ripening, acceleration of leaf senescence by ethylene has received commercial attention in tobacco curing, and celery blanching. Rossi (1934) reported that ethylene reduced the curing time for tobacco by 40%. Harvey (1925) observed that ethylene decreased the chlorophyll content of celery stalks although yellow and red pigments were not affected, and the effect was irreversible. While the acceleration of senescence induced by ethylene with respect to ripening is often considered beneficial, it is harmful when it results in early senescence of vegetative tissue.

This occurs when head lettuce is exposed to minute amounts of ethylene (1 ppm, or less). Thus exposed, lettuce shows reddish brown or olive spots within a few days. This disorder is frequently serious and is known as russet spotting. It affects the appearance and marketability of head lettuce (Rood, 1956). Ethylene could induce similar damage in other green tissues either in an artificial environ (storage chambers, etc.) or in a natural habitat (Phan, 1971).

2. Senescence and biochemical changes

Harvey (1915) demonstrated that etiolated peas, when gassed with 100 ppm ethylene for 72 hours, increased its soluble compounds such as sugars, amino acids and amides with a concomitant decrease in insoluble compounds such as starch, proteins, and cellulose. These observations are typical of senescent tissues and it was therefore concluded that ethylene probably enhances senescence.

Senescence also involves the loss of RNA (Abeles *et al.*, 1967; Sacher and Salminen, 1969; Steffens *et al.*, 1970) and other constituents of the leaf. The effect of ethylene on these degradation processes has been measured in bean plants and the data show that actually RNA degradation was small compared to the reduction of protein levels (Abeles *et al.*, 1967). Sacher and Salminen (1969) performed similar experiments and concluded that ethylene decreased the rate of RNA and protein synthesis in bean pods but had no effect on *Rhoeo discolor* leaf sections.

When excised leaves are kept in light or darkness, there is a rapid breakdown of protein in the blade, accompanied by a loss of chlorophyll. Martin and Thimann (1972) reported that when the first few leaves of oat seedlings were detached and left to senesce in the

dark, the sequence of events was as follows: within a few hours (at 25°C) proteolysis began and about 18 hours later, the chlorophylls began to decrease. After three days, 55-60% of the chlorophyll and about 60% of the total protein had disappeared. Cycloheximide arrested both processes but chloramphenicol did not. Similar results were recently reported with barley leaves by Peterson and Huffaker (1975). In both cases, it was concluded that hydrolytic enzymes, synthesized in the cytoplasm, initiated the senescence phenomenon and the chloroplasts subsequently became involved. The idea was also proposed earlier by Choe and Thimann (1974) who studied the senescence of isolated chloroplasts from oat leaves. They found that the rate of loss of chlorophyll was only about 10% of that which occurred in the detached oat leaves at the same temperature, whereas the loss of protein, though slightly more rapid, was only 36% after seven days. The unexpected stability of the chloroplasts is marked by their photosynthetic activity, 81% of photosystem I and 35% of photosystem II being present after three days at 25°C. Traces of system I, but not of system II, were detectable even after seven days.

3. Ethylene leaf abscission and degradation of chlorophyll

Leaf yellowing is often associated with abscission although a number of examples have been published dealing with acceleration of abscission at high ethylene levels without concomitant yellowing (Zimmerman *et al.*, 1931). While abscission appears to be separated from yellowing, factors such as IAA or CO_2 that block or prevent ethylene action also prevent or delay yellowing. On the other hand, the compounds that have been linked to increased ethylene production have been found to enhance chlorophyll degradation. Foliage sprayed with

preparations of n-butyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D) or of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) becomes pale green or yellow within a few days. The synthetic auxins cause premature senescence and leaves fall probably because of an increase in ethylene production. It is generally accepted that the defoliant action of auxins is mediated through increased amounts of ethylene in the blade (Osborne, 1967, 1968).

4. Degradation of chlorophyll during senescence

The accelerated coloring of fruits is a well-known effect of ethylene (Harvey, 1926; Wolfe, 1931; Denny and Miller, 1935; Gane, 1937; Heinze and Craft, 1953; Pratt and Workman, 1962; Lyons and Pratt, 1964).

The biochemistry of chlorophyll degradation is associated with the destruction of chloroplast (Thomson, 1969). Looney and Patterson (1967) reported an increase in chlorophyllase activity in ripening apples and banana, suggesting that this enzyme plays a role in chlorophyll degradation. However, many other enzymes must also take part in the breakdown of the chloroplasts. The exact nature of these enzymes is unknown. It appears likely that protein synthesis is involved in their appearance (Abeles *et al.*, 1967).

B. Chloroplast Ultrastructure and Function

In a photosynthetic cell, the active chlorophylls are functionally organized in association with specific enzymic components within lamellar membranes. The overall morphology of chloroplasts may vary considerably depending on the organism studied.

Spinach chloroplasts contain primarily two kinds of membrane -

one that forms the boundary of the chloroplast, the "envelope membrane", and the other the "lamellae membranes" which are present inside the chloroplast. The stroma of the chloroplast is the proteinaceous matrix that is enclosed by the chloroplast envelope. In addition to the internal chloroplast lamellar system, it contains a number of particulate structures visible in an electron microscope. These structures include ribosomes and strands of DNA. It is well documented that both these components play a role in chloroplast self-regulation and replication (Kirk, 1970; Sager, 1972). Ellipsoidal starch grains ranging in size from 0.2 μ up to 1.5 μ often lie between the internal lamellae of the chloroplast. Occasionally osmiophilic (lipid) bodies can also be seen.

1. The Chloroplast envelope

The chloroplast envelope is comprised of a continuous double membrane with a subunit structure in thin-sectioned preparations (Weier *et al.*, 1965, 1966a). Numerous studies with isolated whole chloroplasts indicate the plastid envelope acts as a selective barrier to the transport of various metabolites into or out of the chloroplast. In addition, the plastid envelope is thought to play a role in the formation of new internal lamellae. Much of the early literature describing vesicle formation and fusion of the envelope was reviewed by Menke (1962), who concluded that there is no doubt that thylakoids can arise from the inner membrane of the plastid. Recent reports describe a variable pattern of differentiation along the length of the internal chloroplast lamellae suggesting that a complex process of development must exist within the plastid (Park and Sane, 1971).

2. Lamellae (thylakoid) membranes

The internal lamellar structure of high plant chloroplasts has been studied with the electron microscope in numerous laboratories. A characteristic feature is the presence of small membranous disks, called grana, which are stacked one upon another much like a pile of coins (Rabinowitch, 1956). Each granum stack is composed of two or more saclike disks termed thylakoids. It has been recognized for many years that grana stacks are interconnected by membranous regions (the stroma lamellae) that unite thylakoids in separate stacked regions. These have been discussed and compared by Kirk and Tilney-Basset (1967) and more recently by Park and Sane (1971). The view that has emerged from these studies is that each stroma lamella is continuous with numerous thylakoids within the same grana stack and also with thylakoids in different grana stacks (Heslop-Harrison, 1963; Wehremeyer, 1964; Weier *et al.*, 1966b; Paolillo, 1970).

Stroma and grana lamellae of higher plant chloroplasts constitute an elaborate infrastructure of membranes where the conversion of light into chemical energy takes place. These two types of membranes have been separated physically by differential centrifugation following breakage of class II chloroplasts with digitonin, Triton X-100 or a French pressure cell (the stroma lamellae are lighter and smaller) and comparative studies of their structure, gross chemical composition, patterns of development, and enzymatic capabilities have been made (Allen *et al.*, 1972; Arntzen *et al.* 1969; Boardman and Anderson, 1964; Goodchild and Allen, 1973). The biogenetic relationships between the two types of membranes, if any, have not been established clearly.

The stacked membrane (grana) differ in their composition from

unstacked ones (stroma lamellae). The Chl a/Chl b ratio of stroma lamellae (8.1) is higher than grana lamellae (2.7) for spinach leaf chloroplasts (Gasanov and French, 1973). Stroma lamellae are also relatively deficient in Cytochrome 559 and manganese, but are rich in the reaction centre chlorophyll of PS I (P_{700}) as compared to grana lamellae. Electrophoresis of sodium dodecyl sulfate solubilized membranous proteins shows that stroma membranes contain a predominance of the protein complex corresponding to PS I, while grana contain complexes for both PS I and PS II (Sane and Park, 1971).

3. Localization of chlorophyll molecules in thylakoid membranes

A unique feature of chloroplast thylakoids is the high proportion of specialized lipid molecules, chlorophylls, carotenoids and plastoquinones which are not involved in formation of the bilayer but have a functional role (Anderson, 1975a). Because of their prime importance in the capture of light energy, the chlorophylls are the most important component and comprise about 20% of the lipid soluble mass.

As expected of light receptors, which are to be membrane bound, these molecules are amphipathic (Fig. 1A, Anderson, 1975b). The chlorin ring has an area of enhanced polarity in the carbonyl group of isocyclic ring V and in the propionic acid side chain of ring IV, thus giving the square planar ring a hydrophilic edge adjacent to the phytyl tail: the Mg^{++} also has hydrophilic properties. Phytol, a C-20 alcohol composed of four isoprenoid units, has only one double bond far removed from the "tail" end and is thus more rigid than the bulk of chloroplast membrane fatty acids. Chlorophyll b is slightly more polar than chlorophyll a, since it has a formyl instead of a methyl

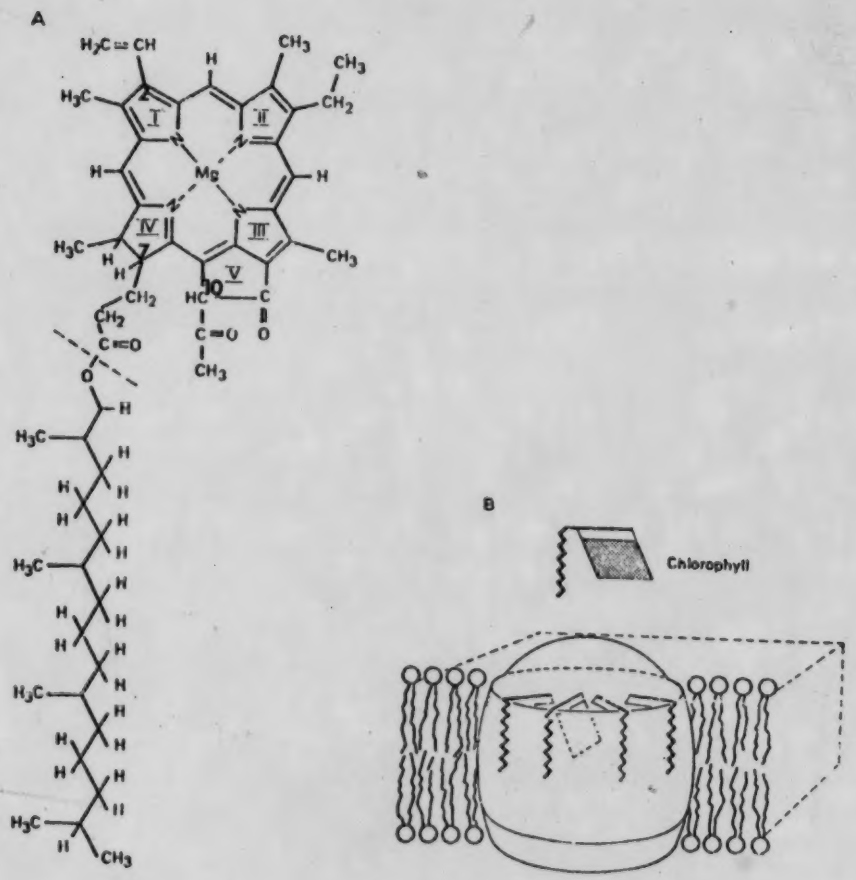


Fig. 1. Possible location of chlorophyll within chloroplast membranes.
A. Chlorophyll a.
B. Schematic cross - section of chloroplast membrane showing an intrinsic protein spanning the membrane, with hydrophilic regions located at the membrane surfaces and a hydrophobic portion (shaded) embedded within the non-polar interior of the lipid bilayer. It is postulated that the chlorophyll molecules (represented above with the hydrophobic portion of the chlorin ring shaded) are located as part of the boundary lipid of a chlorophyll-protein complex. The phytyl chains are perpendicular to the membrane surface in close interaction with the outside perimeter of the hydrophobic region of the intrinsic protein. In contrast, the chlorin rings are buried within the protein: the hydrophilic edge of the chlorin ring is located at the membrane and the hydrophobic part is buried within the hydrophobic interior of the intrinsic complex (from Anderson, 1975b).

group in ring II.

Most of the chlorophyll is likely to be attached to these complexes *in vivo* as part of the boundary lipid, with the chlorin rings located towards the membrane surface (Fig. 1B, Anderson, 1975b). The fact that, in the absence of water, chlorophyll cannot be extracted from leaves or cells with non-polar solvents has often been thought to indicate that the bulk of it is attached to protein *in vivo* (Rabinowitch, 1956). The complexity of *in vivo* spectra strongly suggests that the chlorophylls occur in several environments that arise from interactions of chlorophyll molecules with other chlorophyll molecules (as a result of specific aggregation of the chlorin rings), with proteins and with lipids (Brown, 1972; Katz and Norris, 1973; Kreutz, 1970; Strouse, 1974). Where are the phytyl chains anchored in the membrane to achieve such an orientation? Anderson (1975a) suggested that the phytyl chains are placed adjacent to the outside perimeter of the hydrophobic portion of two major intrinsic proteins, in contact with proteins on one side and lipid on the other, and thus are part of the boundary lipid of the two chlorophyll-protein complexes. The hydrophilic edge of the chlorin ring, adjacent to the phytyl group, interacts at the membrane surface with the exposed hydrophilic segment of the intrinsic protein and the more hydrophobic portion of the tetrapyrrole moiety extends further into the membrane and is buried in the hydrophobic region of the intrinsic protein. Hence, both the phytyl chain and the chlorin ring interact with protein. A schematic diagram of this concept is shown in Fig. 1B (Anderson, 1975b). Additional lipids are needed to complete the boundary lipid layer. The necessary, but undefined aggregation of the chlorin rings required for maximum energy transfer are possible

in this model. Further, this orientation of the chlorin rings so that they are almost buried in the folds of the protein should ensure that the chlorophyll molecules are securely locked in position and might explain why chlorophyll is still associated with proteins, after careful sodium dodecyl sulfate solubilization of the membrane (Thornber, 1975; Anderson, 1975a).

It is well known that chlorophyll a exists *in vivo* as several forms with distinct absorption spectra (Brown, 1972). It is generally believed that two photosystems function in green plant photosynthesis. One photoreaction (PS II) is closer to the oxygen evolving step and mainly utilizes visible light of wavelength shorter than 680 nm absorbed by such active pigments as chlorophyll b, and certain forms of chlorophyll a. The other (PS I) is closer to NADP reduction and is mediated preferentially by the longer wavelength forms of chlorophyll a.

4. Senescence and ultrastructure of chloroplasts

(i) Natural changes

Ikeda and Ueda (1965) followed the changes in chloroplast structure in the mesophyll cells of detached *Elodea densa* leaves floated on distilled water in the dark. As the leaf yellowed, the chloroplasts became swollen and more spherical, the grana and stroma thylakoids became indistinguishable and the osmiophilic globules increased in size. In the final stages, when no obvious trace of chlorophyll remained, the osmiophilic globules still contained carotenoids and were yellow in color. A similar pattern of breakdown has also been observed for detached wheat leaves, floated on water at low light intensity

(Shaw and Manocha, 1965) and in mesophyll cells of kidney bean leaves senescing under natural conditions, while still attached to the plant (Barton, 1966). In senescening bean leaves the first sign of changes was a localized swelling of the chloroplast thylakoids. This was followed by an increasing loss of thylakoids, disappearance of the stroma, and, again the marked accumulation of globules. The outer envelope remained intact. Butler (1967) compared the course of events in the mesophyll cells of both attached and detached cotyledons of cucumber (*Cucumis sativus*). The overall pattern of breakdown bore some resemblance to that in wheat and bean leaves. The first observable difference in this case appeared simultaneously in the chloroplasts and the ribosome population. Chloroplast breakdown results in a spectacular accumulation of osmiophilic globules that were finally released into the ground plasm.

The pattern of changes found in the chloroplasts of the detached first true leaf of cucumber seedlings is slightly different from the pattern found in their cotyledons (Butler and Simon, 1971). The first detectable change is the disappearance of the chloroplast ribosomes, to be followed shortly by the disorganization of the stroma thylakoids and then the grana. Frequently these are replaced by a more simple lamellar system orientated along the long axis of the chloroplast. This, too, later disintegrated with the usual accumulation of osmiophilic globules. A similar pattern of changes has also been reported in disks cut from leaves of brussels sprouts and floated on water in the dark (Dennis *et al.*,

1967).

A further variation on the sequence of chloroplast changes is evident in the yellowing leaves of *Nicotiana rustica* (Ljubestic, 1968). In this instance, as in cucumber leaves, the stroma thylakoids degenerate before the grana, which are still intact when half the original content of chlorophyll has disappeared. At this time, no simple lamellar system is formed and the osmiophilic globules increase in number and size as the grana gradually degenerate. A similar sequence has been observed in cells from the fronds of *Lemna gibba* (Butler and Simmon, 1971) and leaves of *Elodea* (Yoshido *et al.*, 1969). Brief reference is also made in the literature to the degeneration of chloroplasts in leaves of *Xanthium* (Osborne, 1968). Woolhouse (1967) also commented that in leaves of *Perilla* the chloroplasts appear to break down before there is any obvious change in the structure of mitochondria.

(ii) Osmiophilic globules

The most conspicuous change in green tissue is the breakdown of the chloroplasts with its attendant massing of osmiophilic globules. Small globules of this type are regarded as a normal feature of the chloroplast stroma (Gramick, 1961; Menke, 1962, 1966).

There is some evidence that the globules are an accumulation of membrane breakdown products as suggested by Ikeda and Ueda (1964) and by Barton (1966). Lichtenthaler (1969c) showed that the breakdown of chloroplast thylakoids in a

number of species is accompanied by an increase in the levels of carotenoids and lipoquinones in the enlarging globules. In addition, senescing chloroplasts showed a striking resemblance to those chromoplasts which differentiate from normal chloroplast. Thomson (1966) reported that the chloroplast-chromoplast transition in Valencia oranges is accompanied by the formation of the small paragranal globules similar to those found in the chloroplasts of senescing leaves. Indeed, Thomson suggested that they may contribute toward the formation of the large globules in the mature chloroplast. These pigmented oranges have the ability to regreen under natural conditions. In this process the chromoplasts revert back to chloroplasts, the globules disappearing as the thylakoids reform (Thomson *et al.*, 1967). The authors suggest that the lipid from the globules is utilized during membrane formation.

In the development of yellow Narcisus petals the chloroplast thylakoid system becomes rearranged to a system of concentric lamellae (Nichols *et al.*, 1967). However, there is no stage of thylakoid degeneration and no marked accumulation of globules. Finally, the degeneration of the prolamellar body (Tubular lattice structure in young plastids) on the exposure of young leaves to light is accompanied by an increase in the number of globules, possibly as breakdown products of its lipoprotein membrane (Gunning, 1965).

The chloroplast globules from a number of plants (Bailey and Whyborn, 1963; Barr *et al.*, 1967) have been isolated and found to comprise a mixture of lipids and other substances, not all of which are

to be found in chloroplast membranes. The composition of the globules changes with age and it may well be that the globules act as a general store or reservoir of excess lipids and other substances (Lichtenthaler and Pevelling, 1967).

Obviously there is no single explanation for the marked accumulation of globules in senescing chloroplasts. However, the evidence indicates that they consist of both membrane breakdown products and the still accumulating precursors. They may also represent a general reservoir of excess insoluble lipid material not necessarily connected to membrane breakdown (Bailey *et al.*, 1966).

(iii) Protein

When leaves senesce the loss of chlorophyll is accompanied by disappearance of a large proportion of the protein that was originally present. Since most of the protein in green leaves is located in the chloroplasts (Zucker and Stinson, 1962), a leaf cannot suffer much protein loss without harm to its chloroplasts. In wheat leaves (Shaw and Manocha, 1965; Shaw *et al.*, 1965) and in cucumber cotyledons (Lewington *et al.*, 1967; Butler, 1967), the loss of chlorophyll and protein were correlated with changes in chloroplast ultrastructure.

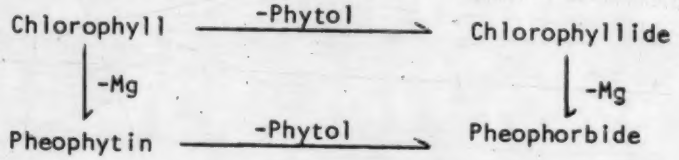
It may well be that the loss of protein from chloroplasts is the factor that initiates chloroplast breakdown, accounting for the similarity in the symptoms shown by chloroplasts in tissues that are senescing.

C. Chlorophyll Degradation

1. Structure and major functional groups

A common feature of the chlorophylls, distinguishing them from

nonphotosynthetic porphyrins is the cyclopentanone ring, conjoining them with ring III (Fig. 1). All naturally occurring porphyrins have a propionic acid residue at position 7. In the chlorophylls, this position is esterified with a long-chain alcohol (phytol, C_{20}). The phytol free compound is known as a chlorophyllide; if it does not contain magnesium, it is a pheophorbide. In the form of the naturally occurring ester, the compound is a pheophytin.



Chlorophyll can be compared to a tadpole, with a porphyrin "body" and a phytol "tail". The "tail" makes up about one-third of the molecule by weight. The lipophilic phytyl group is present in every functional chlorophyll. It is probably responsible for the association of chlorophylls with lipoproteins or carotenoids of thylakoid membrane in nature (also see page 8).

The alicyclic ring V, with its keto oxygen function and carbomethoxy group and the 7-propionic ester group constitute the most hydrophilic part of chlorophyll. In a monolayer over water, chlorophyll sits with these groups against the water, the phytol group and the rest of the porphyrin part folding into a V-shape at an angle to the water surface (Bellamy, 1963). This property of chlorophyll molecules conforms with the model of chlorophyll location in thylakoid membranes as proposed by Anderson (1975b). The ester bond between 7-propionic acid and phytol will be exposed to the surface of the membrane and would be subjected to possible hydrolysis by the chlorophyllase

(chlorophyll-chlorophyllide-hydrolase, EC 3.1.1.14).

Finally, chlorophylls are chelate compounds of magnesium. Unlike the case of metal porphyrins in nature, in which the metal is always a transition element with multiple oxidation states (*e.g.* Fe), no obvious role for the magnesium has been suggested. Some earlier workers believed that the central magnesium atom was somehow involved in the activation of chlorophyll fluorescence (Evstigneev *et al.*, 1950; Livingston, 1960), but no detailed description was available. Recent IR and NMR investigations (Katz *et al.*, 1966), however, have provided strong experimental support for the view that the coordination properties of magnesium in chlorophyll are of decisive importance in determining the state of aggregation of chlorophyll. Spectroscopic investigations lead to the conclusion that magnesium in chlorophyll with coordination number four is coordinatively unsaturated, and that one or both of the magnesium axial positions must always be occupied by an electron donor group (Katz, 1972). Thus, despite authoritative statements to the contrary (Rabinowitch and Govindjee, 1969), the part played by magnesium in chlorophyll behavior is rapidly being clarified and the details can be seen in the excellent review of Katz (1972).

2. Possible mechanisms of chlorophyll degradation in plants

There are four major mechanisms involved in degradation of chlorophylls.

(i) Removal of Mg^{++} by H^{+} to form pheophytin

The central Mg ion of chlorophylls is readily displaced by strong or weak acids (Willstätter and Hocheder, 1907), a phenomenon known as pheophytinization. The greater stability of chlorophyll b to pheophytinization compared with chlorophyll a was explained by the

following mechanism (Cho, 1966):

$$Chl + 2H^{+} \underset{}{\overset{keq}{\rightleftharpoons}} [(Chl\ H_2)^{++}] \xrightarrow{K_2} \text{Pheophytin} + Mg^{++}$$

in which the rate is determined by Keq. The Keq for chlorophyll b is about ten times smaller than for chlorophyll a because of resonance structures. These structures place a greater positive charge on the nitrogen atoms of chlorophyll b that are attacked by protons than on the equivalent nitrogen atoms of chlorophyll a.

The presence of chlorophyll a and b in the leaves of higher plants has focused attention on their comparative biochemistry. Their behaviour during senescence has been investigated at various times, and it appears that chlorophyll a generally tends to be destroyed at a faster rate (Seybold, 1943; Jeffrey and Griffith, 1947; Wolf and Wolf, 1955) than chlorophyll b.

Wolf (1956), in a comparative study of the leaves of twenty-five species of trees, showed that chlorophyll a averaged 69.4% of the chlorophylls in green leaves and 56.2% in yellow leaves. However, this is contrary to the previously held view that the ratio remained constant during the fading of autumn leaves (Goodwin, 1952). Weybrew and Mann (1958) reported earlier that the ratio of Chl a/Chl b of senescent tobacco leaves does not fall appreciably until most of the chlorophyll has already disappeared. Recently, Whitfield and Rowan (1974) also found that the Chl a/b ratio of *Nicotiana tabacum* leaves decreased during senescence only in post-mature leaves. It is possible that the disintegration of vacoules (with high acid content) of plant cells of green tissues (Butler and Simon, 1971) during late senescence may play an important role in pheophytinization. The real mechanism of degradation of chlorophyll during senescence is unknown.

The difference in reactivity of chlorophyll a and b has been noticed in the making of silage, where the chlorophylls differ in their resistance to destruction by acids (Wolf, 1956) and similar results during drying of plant materials have suggested that acids normally present in leaves are important for the destruction of chlorophyll. The first manifestation of acid modification of chlorophyll should be pheophytin formation, and this is often found in leaf extracts although it is not known whether it is an artifact of extraction. Pheophytin formation during processing and storage of fruits and vegetables is, however, an observed fact (Chichester and Nakayama, 1965)..

It is interesting to note that pheophytinization of chlorophylls results in grey color that can be regreened by the chelation of metallic ions; *e.g.* Cu and Zn (Schanderl *et al.*, 1965). Magnesium, while easily removed from the porphyrin ring, is difficult to replace.

(ii) Alteration of the porphyrin ring

Strain and Manning (1942) were the first to report the presence of minor components in a chlorophyll mixture when chromatographed on powdered sugar columns. They showed that these minor components are slightly less absorbed than the respective chlorophylls a and b, and were designated chlorophyll a' and b'. Katz *et al.* (1968) have obtained evidence from proton magnetic resonance spectra of chlorophylls a' and b' showing that these compounds are C_{10} epimers of the respective chlorophylls. The chlorophylls a and b were converted into chlorophylls a' and b' when plant leaves or leaf extracts were exposed to treatments that included heating (Strain, 1954; Bacon and Holden, 1967) and the action of organic solvents (Pennington *et al.*, 1964; Bacon and Holden, 1967). From heat and organic solvents treatment,

Bacon and Holden (1967) have found four more so called "changed chlorophylls a_1, a_2 and b_1, b_2" with different TLC characteristics and slightly different absorption spectra. They proposed that the "changed chlorophylls a and b" also arise from the alteration of the components at C_{10} of respective chlorophylls.

A mechanism for the nonenzymatic breakdown of chlorophyll which involves oxygen attack on the isocyclic carbon C_{10}, was proposed by Chichester and Nakayama (1965). They presumed that the process involves the oxidation of C_{10} followed by a fission of the ring to form a variety of purpurins and chlorins. These compounds are found in moderate quantities in dried plant materials (Aronoff, 1953). The exact pathway of oxidation is not known *in vivo*, and is only guessed at *in vitro*. The further oxidation of porphyrins and chlorins occurs through complete scission of the isocyclic ring, followed by oxidation of the tetrapyrrols (Aronoff, 1953).

(iii) Loss of chlorophyll in relation to lipoxidase activity

The loss of chlorophyll in frozen peas has been related to lipoxidase action as proposed by Wagenknecht and Lee (1956, 1958) and Lee and Wagenknecht (1958). Walker (1964) found that chlorophyll degradation in frozen beans was related to fat peroxidation in a manner analogous to the carotenoid degradation.

Holden (1965) made an extensive study on chlorophyll bleaching in a system containing a fatty acid, lipoxidase and a legume seed extract. Chlorophyll was rapidly bleached in this mixture and no pheophytins, chlorophyllides or pheophorbides were detected. Neither peroxidized linoleic acid nor purified lipoxidase plus lipid bleached the chlorophyll as rapidly as the crude extracts. Therefore, she

postulated that a co-oxidation factor for chlorophyll loss is probably present in crude extract. Oxygen was necessary for the reaction; anti-oxidants inhibited the reaction but cyanide did not.

The coupled oxidation of chlorophyll with linoleic acid catalyzed by lipoxidase was also reported recently by Kies *et al.* (1969), Buckle and Edwards (1970), Orthoefer and Dugan (1973) and Imamura and Shimizu (1974). No evidence was provided for the participation of hydroperoxide isomerase (Imamura and Shimizu, 1974). Zimmerman and Vick (1970) demonstrated that chlorophyll is bleached only in the presence of linoleic acid lipoxidase and hydroperoxide isomerase. However, Imamura and Shimizu (1974) failed to observe any evidence for the participation of hydroperoxide isomerase.

The amount of free fatty acids in plant leaves is usually small, but the first stage might be their liberation from lipids by lipases, thus providing the substrate for lipoxidase. For example, linoleic acid will be formed from mono- and di-galactosyldiglycerides by galacto-lipases. The activity of these enzymes is low in spinach leaves (Sastry and Kates, 1964). Because of this, little substrate will be available for the lipoxidase, therefore the formation of hydroperoxide for degradation of chlorophyll will be blocked.

(iv) Hydrolytic detachment of the phytol group catalyzed by chlorophyllase

The question whether chlorophyllase is an enzyme in the pathway of chlorophyll biosynthesis or degradation of both is another topic that is pertinent to the mechanism of chlorophyll degradation. There is evidence in support of both synthetic (Böyer, 1965; Chiba, 1967; Hines and Ellsworth, 1969; Holden, 1961: Shimizu and

Tamake, 1962; Stobart and Thomas, 1968; Sudyina, 1963) and degradative (Looney and Patterson, 1967; Peterson and McKinney, 1938; Rhodes and Wolltorton, 1967; Ziegler and Schanderl, 1969; Sivtsev *et al.*, 1973) roles for chlorophyllase. Even so, two forms of the enzymes, chlorophyllases, were believed to be in the leaves. Recently, Ellsworth (1971, 1972a) reported that the chlorophyllase from wheat seedlings catalyzed hydrolysis of pheophytin a, transphytylation of methyl pheophorbide a to pheophytin a and phytylation of pheophorbide a to pheophytin a. He found that these activities were affected differently by changing the reaction conditions such as acetone concentration, buffer concentration, pH and temperature. From these findings, he suggested the possible involvement of more than one enzyme. Further results, presented in his subsequent paper (Ellsworth, 1972b) on gel filtration and acetone fractionation of the enzyme, provided additional evidence to support his postulation. Similar results were also obtained from sugar-beet leaves (Bacon and Holden, 1970), tea leaves (Ogura, 1969, 1972), *Ailanthus altissima* (Tree-of-Heaven) leaves (McFeeters *et al.*, 1971), endive and spinach leaves (Terpstra and Weijman, 1972) and *Chlorella* protothecoides (Ichinose and Sasa, 1973).

The chlorophyllase of sugar-beet leaves was characterized and partly purified by gel filtration and ion-exchange chromatography on various types of Sephadex (Bacon and Holden, 1970). Indications of more than one form of the enzyme may be explained by its tendency to associate or aggregate with itself or with other substances. The enzyme is strongly adsorbed to negatively charged materials, which may explain its improved extractability when the sodium chloride concentration of the medium is increased.

Soluble and insoluble chlorophyllases were isolated from tea leaves by Ogura (1969, 1972). Soluble chlorophyllase was partially purified by procedures including ammonium sulfate fractionation (preparation I). The insoluble fraction was extracted, by solubilizing it with sodium deoxycholate. This initial insoluble enzyme was also partially purified (preparation II). Specific activities (mg chlorophyll a hydrolyzed per hour per mg protein, 7.2 for preparation I and 12.4 for preparation II), were much higher than those reported for enzyme preparations from other plant material. The soluble enzyme was more resistant to PCMB (p-chloromercuribenzoate), lipase and heat treatment than the insoluble enzyme. The two enzymes differed in optimum temperature and optimum acetone concentration needed for the reaction, but showed the same optimum pH, and same K_m value (7 μM). These results suggest that, in spite of differences in location and extractability, activities of the soluble and insoluble (solubilized) chlorophyllase in tea leaves are attributable to the same enzyme.

Chlorophyllase from *Ailanthus altissima* leaves has been purified 63-fold by a combination of heat treatment, ultracentrifugation, gel filtration and chromatography on diethylaminoethyl cellulose. The enzyme was found to have a pH optimum on pheophytin a of 4.5. The chlorophyll a and b, pheophytin a and b, and pyropheophytin a were hydrolyzed by the enzyme while protochlorophyll a and 4-vinyl protochlorophyll a were not hydrolyzed but were competitive inhibitors of the enzyme. They also claimed that the enzyme did not appear to contain an essential sulfhydryl group since sodium tetrathionate and PCMB did not affect its activity.

Chlorophyllase was extracted from green cells of *Chlorella*

protothecoides by n-butanol treatment and purified 600-fold (Ichinose and Sasa, 1973), as measured by enzyme activity in chlorophyll a hydrolysis, by ammonium sulfate precipitation, chromatography on a TEAE-cellulose column and gel filtration with Sephadex G-200. At each purification step the following activities were compared: hydrolyses of chlorophyll a and methyl chlorophyllide a, methanolysis of chlorophyll a and transphytylation of methyl chlorophyllide a to chlorophyll a. The ratio of activities of chlorophyll a hydrolysis to chlorophyll a methanolysis changed on purification and partial inactivation by heat, PCMB and phytol, as well as by varying the reaction temperature, thus suggesting that the two reactions are not catalyzed by a single enzyme. Results of kinetic studies also indicated that the chlorophyllase of *Chlorella protothecoides* consists of at least two enzymes. One enzyme catalyzes chlorophyll a hydrolysis and the other, chlorophyll methanolysis and the reverse reaction, transphytylation of methyl chlorophyllide a.

Spinach leaves contain a "Spinach Protein Factor" (SPF) which increases light sensitivity of colloidal chlorophylls in aqueous solution (Terpstra, 1967). SPF activity, measured in different fractions of spinach-leaf acetone-powder extracts obtained by gel filtration on DEAE- and CM-Sephadex, runs parallel with chlorophyllase activity. The same positive correlation is generally observed in aqueous extracts of certain small particles isolated from spinach-leaf homogenates. It is suggested that SPF is a chlorophyllase (Terpstra and Weijman, 1972). Spinach, then, may contain two kinds of chlorophyllase, a very active one which shows both chlorophyllase and SPF activity, and a less active one, showing only chlorophyllase activity.

Only the latter form occurs in endive.

(v) Chlorophyllase and degradation of chlorophyll during storage

Degradation of chlorophyll during the storage of plant tissues is still a completely unsolved problem despite the fact that loss of chlorophyll is commonly used as a measure of senescence. Hoyt (1966) subjected chopped ryegrass (*Lolium perenne*) to a number of different treatments and measured chlorophyll loss. Conditions of high humidity and normal temperatures resulted in rapid chlorophyll degradation. Freezing, boiling, dessication and waterlogging all stopped chlorophyll loss. Cooling slowed the degradation, but it returned to normal after rewarming. These data are consistent with an enzymatic mechanism of degradation. There is some preliminary evidence that chlorophyllase does not catalyze the initial step of chlorophyll degradation in cur ryegrass (Chichester and McFeeters, 1971). It is not possible to say that chlorophyllase is not involved in chlorophyll degradation because it may catalyze removal of phytol group at a later step in the degradative pathway. The first step in the degradation requires the presence of oxygen, but it is not known whether oxygen is directly involved in the reaction or whether it is needed indirectly for maintenance of the degradative system (Chichester and McFeeters, 1971). Holden (1972) reported that bleaching of chlorophyll is clearly linked with the degradation of protein and probably also of lipids of chloroplast membranes. She further mentioned that when photosynthesis is inhibited fully, the chloroplasts became non-functional and catabolic reactions associated with senescence began, leading ultimately to death.

D. Present Investigation

The importance of maintaining green color in green fruits and vegetables and in the leaves of other plants need not be over emphasized. If the green color of some fruits and vegetables is lost the products become unacceptable to the consumers and thus results in an economic loss to the growers. Similarly, if the green leaves of a plant begin to lose their color, the plant will soon be unable to photosynthesize and die eventually.

This process of degreening is a common phenomenon in both detached and attached plant tissues, *e.g.* leaves, but the inducing factor(s) and underlying mechanism(s) are still unclear. Ethylene was implicated with senescence and concomitant yellowing of potato leaves by Denny and Miller (1935). Since then, various other workers have obtained a similar effect of ethylene on other plant species and it is now widely accepted that ethylene is a causative agent for triggering senescence and yellowing in plants.

In addition to the phenomenon of natural senescence and yellowing of plant tissues, unnecessary exposure to abnormal amounts of ethylene often brings about a premature senescence, yellowing and ultimate death of plant tissues. This is an aspect of air pollution where higher than acceptable amounts of ethylene in the air can cause damage to plants.

How does ethylene bring about this change in color, from green to yellow? One looks at the chloroplasts and the chlorophylls which impart green color to the plant tissues and organs, for the answer.

Although there is a considerable amount of information

encompassing the physiological and biochemical aspects of senescence (Varner, 1961; Wagermann, 1965; Woolhouse, 1967 ; Wareing and Phillips, 1970; Sacher, 1973), yet at the ultrastructural level the data are still relatively sparse and scattered. Ikeda and Veda (1964) studied the changes in the structure of chloroplasts of detached *Elodea densa* (pondered) leaves. They found that as the leaves yellowed, the grana and stroma thylakoids became indistinguishable and the osmiophilic globules increased in size and number. Shaw and Manocha (1965) observed similar breakdown of chloroplast membranes in senescing kidney bean and wheat leaves. They found that in senescing bean leaves the first sign of change was a localized swelling of the chloroplast thylakoids. This was followed by an increasing loss of thylakoids, disappearance of the stroma, and, again a marked increase in the osmiophilic globules, but the outer envelope still remained intact.

What triggers this degeneration of chloroplast ultrastructure? In the present investigation, the effects of ethylene on the ultrastructures of chloroplasts in detached spinach leaves have been studied to evaluate the role of ethylene in chloroplast degeneration.

Because chloroplast ultrastructural integrity is very important for maintaining the physiological functions of chloroplasts, the effects of ethylene on the CO_2 assimilation by the detached spinach leaves as well as on the Hill reaction by chloroplasts have also been studied to evaluate the extent of ethylene effect on the photosynthetic activity. As the loss of chlorophyll and degeneration of the chloroplast ultrastructure proceed more or less simultaneously, attempts were made to characterize the degradation products of chlorophyll as well as to study the effect of ethylene on the enzyme, chlorophyllase,

which has been suggested as a possible enzyme responsible for catalyzing breakdown of chlorophyll. Also, the changes in the soluble protein content of detached spinach leaves was investigated to find out the nature of relationship that exist between senescence and protein metabolism. Finally, the effects of so-called ethylene-antagonist, namely CO_2 and some other anti-senescent agents, were studied in an attempt to find a possible means to prevent the degreening of green plant tissues.

MATERIALS AND METHODS

A. Materials

1. Chemicals and supplies

Routine chemicals of reagent grade (meeting ACS specifications) were bought from Canadian Laboratory Supplies, Fisher Scientific Company and Mallincrodt Canada Ltd. The other specific chemicals, 2-(N-morpholino)-ethane-sulfonic acid (MES), N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES), N-tris(hydroxymethyl)methylglycine (TRICINE), Nicotinamide adenine dinucleotide phosphate (NADP), kinetin, N-6-benzyladenine (NBA), bovine serum albumin (BSA), Chloramphenicol, and cycloheximide were obtained from Sigma Chemical Company. Osmium tetroxide and other chemicals used for electron microscopic studies were purchased from Ladd Research Industries, Inc.

2. Plant materials and growth conditions

(1) *Chlorella pyrenoidosa* L.

Chlorella was cultured in a nutrient medium adapted from Gorham (1972). The chemical composition of the medium was as follows: $NaNO_3$, 2000 µM; KH_2PO_4, 100 µM; $Na_2HPO_4 \cdot 2H_2O$, 100 µM; $MgCl_2 \cdot 6H_2O$, 200 µM; $MgSO_4 \cdot 7H_2O$, 200 µM; $CaCl_2 \cdot 2H_2O$, 200 µM; $FeCl_3 \cdot 6H_2O$, 4 µM; $Na_2EDTA \cdot 2H_2O$, 20 µM. One ml of a micronutrient solution which consisted of the following chemicals was added to the above mixture: H_3BO_4, 40 mM; $MnCl_2$, 7 mM; $ZnCl_2$, 3.2 mM; $CoCl_2$, 0.08 mM; $CuSO_4$, 0.008 mM. Sterile conditions were achieved by autoclaving the growth media and gassing system at 1.05 kg/cm^2 pressure for 20 minutes and transfering the *Chlorella* in a flow bench (Edgegard Hood with horizontal laminar flow, the Baker

Company, Inc.).

Carbon dioxide in air stream (0.03%) at a flow rate of 200 ml per minute was continuously bubbled through the medium [200 ml in one litre flask (Bellco)], in which *Chlorella* was grown. The other conditions for *Chlorella* growth were as follows: light intensity, 300 ft candles; temperature, 26°C; pH of the medium, 6.7; and a continuous shaking with 90 strokes per minute.

(ii) *Lemna minor*, L. (duckweed)

The *Lemna* was grown in 50 ml of nutrient solution in a 250 ml wide mouth Erlenemeyer flask under sterile conditions. The nutrient solution contained $MgSO_4$, 2 mM; $Ca(NO_3)_2$, 7 mM; KNO_3, 5 mM; KH_2PO_4, 2 mM; FeNaEDTA, 38 µM: H_3BO_3, 46 µM; $MnCl_2$, 9.2 µM; $CuSO_4$, 3.2 µM; $ZnSO_4$, 1.8 µM; Na_2MoO_4, 4.1 µM; $CoCl_2$, 3 µM and 2-3 crystals of KI per litre of solution (Wong and Dennis, 1973). The pH of the medium was 6.0. The medium was sterilized in the same manner as that for the *Chlorella*. Usually, one plant consisting of three to four fronds was transferred to the growth medium in a flow bench and the cultures were plugged with a de-oiled cotton and grown in a growth cabinet at 26°C and under continuous illumination at 800 ft candles.

(iii) *Spinacia oleracea*, L. (spinach)

Spinach (cv King of Denmark) was grown in a growth chamber under a light intensity of 4400 ft candle (1.13×10^4 watts/ cm^2/) with a combination of fluorescent and incandescent lamps. The spectra of fluorescent and incandescent light are shown in Fig. 2 and Fig. 4, respectively. The relative humidity inside the chamber was 75%. The photoperiod for growth was twelve hours. The temperature for growth was 20°C. Some spinach plants for this study

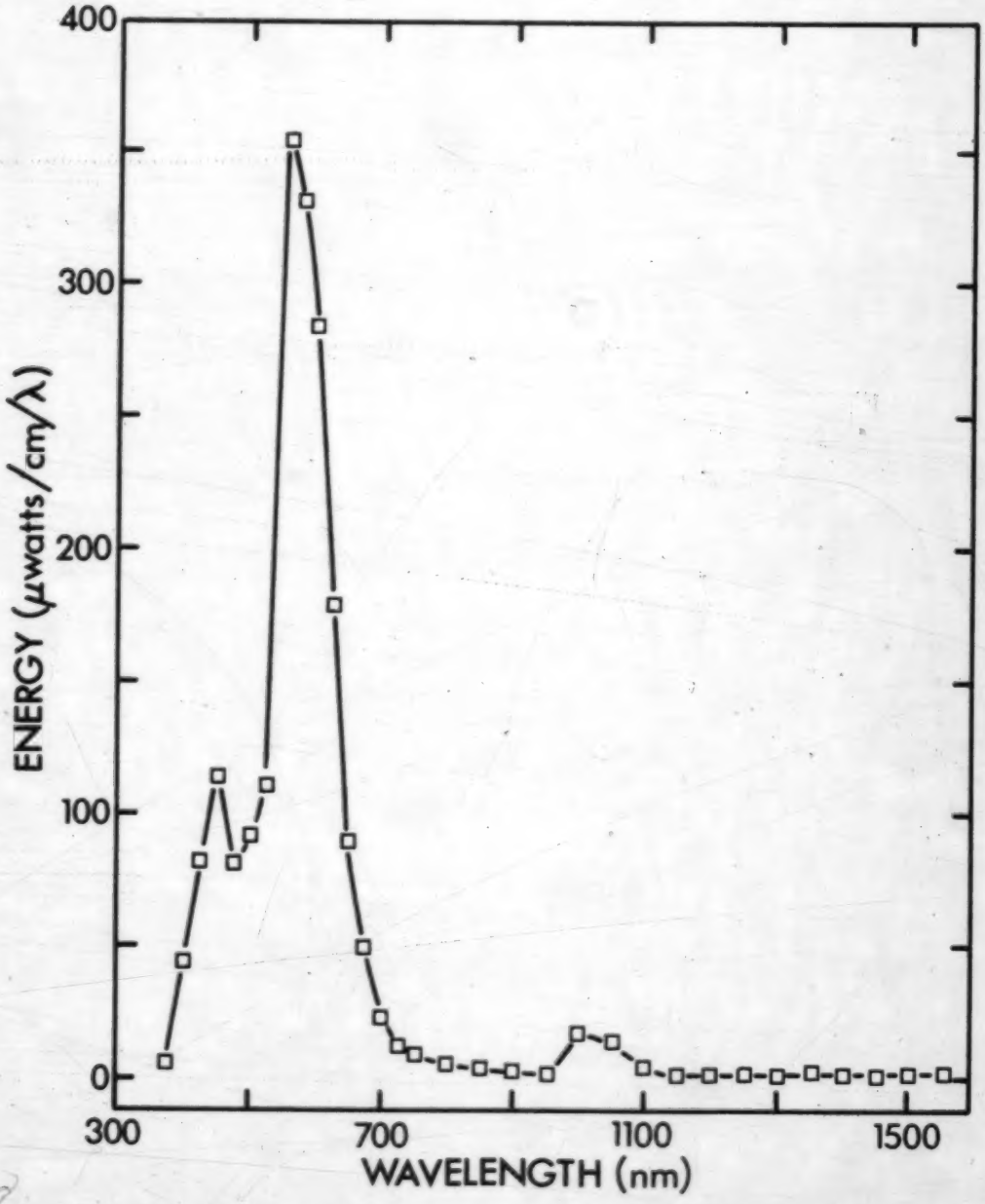


Fig. 2. Spectrum of light from fluorescent lamp (Philips). The light energy was measured by Spectroradiometer, Model SR, Instrumentation Specialities Company, Inc., Lincoln, Nebraska, U.S.A.

were also obtained from the University of Alberta Farm and some were purchased from the City Market.

The fifth leaf from the apex of a spinach plant grown in a growth chamber was chosen for electron microscopic studies.

B. Methods for Chemical Determinations and Enzyme Assay

1. Ethylene determination

(i) Collection of ethylene

The method described by Sarkar (1972) was used. A continuous stream of air, freed of ethylene by passing through mercuric perchlorate trap* in ice, then dried by passing through a Drierite trap**, was allowed to flow at the rate of 200 ml per minute into a jar containing spinach leaves (totalling a weight as close as possible to 10 g) with their petioles dipped into a beaker containing 60 ml of water (or the solution of an assayed compound in order to sweep out the gas mixture evolved from the spinach leaves.

Immediately prior to the collection of ethylene, the glass U-tube containing 1 g silica gel was heated in a boiling water bath for about 30 minutes. During this heating period, a stream of purified nitrogen was passed continuously through the tube to remove all the

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*The ethylene trap was a 1.5 cm inner diameter glass U-tube packed with mercuric perchlorate adsorbed silica gel. This packing was prepared by mixing two parts (by weight) of silica gel (98-200 mesh) with 1 part (by weight) of a solution of mercuric perchlorate. The solution was made by dissolving 56.7 g of $Hg(ClO_4)_2 \cdot 3H_2O$ in 50 ml of water, then adding 43 ml of 70% $HClO_4$, and finally making up to 250 ml with water.

**This trap is a 1.5 cm inner diameter U-tube containing 98 mesh Drierite.

gases that were released from the silica gel (Knight, 1970). The tube was sealed with a short piece of ethylene-free white rubber tubing and transferred to the collection system.

(ii) Estimation of ethylene

Ethylene was estimated by gas chromatography (Varian Aerograph, model 1700) using a 1.5 m x 0.3 cm outer diameter stainless steel column, packed with 60-80 mesh activated alumina (Phan, 1970). The gas from the glass U-tube was directly delivered into the column by means of an auxiliary carrier gas (N_2) flow mechanism. One end of the U-tube was connected to the auxiliary carrier gas flow system (back flush, four point valve) keeping the U-tube in dry ice-acetone bath. The valve was opened for 30 seconds and the U-tube was flushed with nitrogen. After 30 seconds the valve was closed and the other end of the U-tube was connected to nitrogen flow system. With the valve closed the U-tube was heated in a water bath at 50 ± 1°C for 3 minutes to release gases from the silica gel. The valve was then opened and the contents of the U-tube were flushed onto the gas liquid chromatography column. The temperature settings were as follows: column - 50°C, injection port - 125°C, and detector - 200°C. Carrier gas flow rate was 24 ml/minute. The amount of ethylene in an unknown sample was determined from the peak response in the chromatogram which was compared to that of known amounts of standard ethylene in nl. The instrumental error was kept minimal by maintaining the same conditions in all the runs.

Ethylene in the sealed jar containing spinach leaves was determined by injecting the gas sample directly into the column through a septum, at the injection port, by means of a gas tight syringe. The concentration of ethylene was calculated by comparing to

the standard ethylene in ppm.

2. Determination of chlorophylls

The chlorophylls from spinach leaves and *Lemna* were extracted by cold 80% acetone. For homogenization, a Waring blendor was used for spinach leaves (high speed at 4°C for two minutes), and a mortar and pestle in ice bath was used for *Lemna*. After the extraction the chlorophyll content was determined spectrophotometrically at 649 nm and 665 nm for chlorophylls a and b according to the formulae developed by Vernon (1960).

The chlorophylls and other pigments of spinach leaves were extracted with 80% acetone, ethanol-diethyl ether (3:1) and diethyl ether by the method of Bacon and Holden (1970) and then separated by column, and thin layer chromatography (see below). The combined extracts were evaporated almost to dryness in a flask evaporator in the dark at 30°C. The residue was resuspended in a known volume of chloroform. An aliquot was taken for chromatographic separation.

The extraction of chlorophylls from *Chlorella* was more difficult than from spinach leaves or *Lemna*. Mortar and pestle, Vortex homogenizer and sonicator were used to extract chlorophylls; none of these proved satisfactory. Even when *Chlorella* cells were frozen before homogenization, the extraction of chlorophylls with 80% acetone was not complete. Finally, absolute methanol was used as reported by Hess and Tolbert (1967). It gave a complete extraction of chlorophylls. One to ten milliliters of *Chlorella* suspension (depending on the stage of culture) were centrifuged in a Beckman centrifuge, model JB 21, at 2,000 g and 0°C for five minutes. The supernatant layer was discarded and the tube was inverted on an absorbent surface to eliminate excess

water. After several minutes, the cells were resuspended in 3 ml of absolute methanol and placed in a stoppered centrifuge tube in the dark for two hours to insure complete extraction of the pigments. After centrifugation, the absorbance of the green supernatant fraction was measured and the concentration of chlorophyll a and b calculated (Mackinney, 1941).

3. Separation of pigments of spinach leaves by chromatographic techniques

(i) Thin-layer chromatography (TLC)

Two different techniques were used for separation of chlorophylls and other pigments of spinach leaves.

a. Microscope slide TLC

For qualitative analysis and demonstration purposes separation on microscope slides (Rollins, 1963; Bacon 1965) is very advantageous, because while the quantity of applied pigments is small, the development is rapid (80 seconds, Bacon, 1965) and the preparation of layers does not require a spreading device which is necessary for normal standard work.

A microscope slide was dipped in 40% silica gel 7G (Canlab) water solution and then left overnight. The air dried slides coated with silica gel were placed in an oven for one hour at 110°C and the slides were stored in a dessicator until used (Phan, personal communication). The solvent system was 3% methanol in chloroform. The separation was achieved within three minutes.

b. Reversed phase TLC

Egger (1962) first proposed the reversed-phase partition method, later modified by Jones *et al.*, 1972 for TLC separation of

chloroplast pigments to avoid the destruction of pigments when using adsorption chromatography techniques involving inorganic thin layers.

Glass plates (5 x 20 cm) were coated with Kieselguhr G (250 μ) partially impregnated with peanut oil dissolved in heptane (14% peanut oil). Freshly coated plates were found to be satisfactory if permitted to dry in air for sixteen hours or longer before oil-impregnation. After oil-impregnation, the plates were allowed to drain for one hour or longer. Plates were oven dried at 110°C for one hour to volatilize the solvent and to activate the coating.

The development was achieved with a mixture of methanol-acetone-water (20:4:3) in the dark. The antioxidant, butylated hydroxytoluene (BHT) in diethyl ether (150 mg per 100 ml) was added to a working volume of the sample for spotting at a concentration of 0.1% per ml to prevent the oxidation of pigments. It was found necessary to prepare a new batch of antioxidant every month. The spots of different colors could be seen distinctly when viewed under visible or ultraviolet light.

(ii) Sephadex LH20 gel column chromatography

The method described by Shimizu (1971) was used with a slight modification of column size and solvent mixture. Sephadex LH20 gel was swollen in chloroform-methanol (70:30) for twenty-four hours and poured into a glass Sephadex column (Pharmacia SR 25/45, 25 x 45 cm) to a height of about 20 cm. The suspension medium was replaced with chloroform-methanol (95:5) and finally with chloroform only. When the medium was completely replaced with chloroform the color of the column changed from white to semi-translucent white and the height of the column bed increased to about 23 cm. Two flow adaptors were used because the specific gravity of chloroform (1.47) was greater than that

of Sephadex particles (sp. gr. = 1.3). Ascending chromatography was applied. Development with chloroform was continued until the last two bands moved up, a mixture of chloroform-methanol (70:30) had to be used to elute them. It has been reported that the particles of Sephadex LH20 show a specific affinity towards compounds having a -COOH group, in chloroform (Pharmacia Handbook, 1966). The last two bands would be chlorophyllides and pheophorbides having -COOH group. The eluted fractions of the chloroplast pigments from air control and ethylene-treated samples were measured qualitatively and quantitatively by spectrophotometry. The absorption spectrum of each fraction was obtained from a Cary 15 and Perkin-Elmer, model 202 recording spectrophotometers.

4. Chlorophyllase assay

The method of chlorophyllase assay was adapted from Holden (1961) and Bacon and Holden (1970) with some modification. Buffer (8 ml) used by them was found not sufficient to dissolve one gram of acetone powder.

(i) Preparation of crude enzyme

Spinach leaves were ground in a Waring blendor at high speed for five minutes with cold acetone (-20°C) to give a concentration of 80%. The suspension was filtered through a Whatman No. 1 filter paper on a millipore funnel to prevent loss of precipated enzymes. The residue was washed twice with cold ethanol-diethyl ether (3:1) and twice with diethyl ether for complete removal of pigments. The powder was left at room temperature for two hours to ensure the escape of solvent.

A weighed sample of powder (up to 500 mg) was mixed with 0.02 M sodium citrate buffer (pH 7.0) containing 0.4 M NaCl (24 ml per g

powder) and left at 4°C overnight. The extract was squeezed through a layer of miracloth (Calbiochem) and the residue rinsed several times with 0.02 M sodium citrate buffer (pH 7.3) and made to 25 ml. The preparation was centrifuged at 8000 rpm at 4°C for ten minutes and the pellet discarded. Four ml of this clear crude extract was used for chlorophyllase assay.

(ii) Determination of chlorophyllase activity

a. Substrate

The substrate, chlorophylls were prepared by grinding spinach leaves in cold acetone (4 ml per g leaf), leaving the extract at 4°C in the dark for several days and filtering off the precipitated carotenes (Holden, 1961). The solution contained about 20% of water and 0.55 mg per ml of chlorophyll a and 0.26 mg per ml of chlorophyll b as determined by the method of Vernon (1960).

b. Incubation

Four ml of duplicate samples of the enzyme extract were added into two 20 ml screwed tubes which contained 4 ml each of chlorophyll solution. The mixture was shaken and left in the dark at 22 ± 1°C for 30 minutes. One tube was kept at -20°C for determination of the total chlorophylls and chlorophyllides (I) (see below). To the other tube was added about 2 ml of petroleum ether and 2 ml of 80% acetone. The mixture was shaken briefly but vigorously. The upper layer contained chlorophyll (ether layer) and the bottom layer contained chlorophyllide (aqueous layer). The chlorophyllide layer was extracted with petroleum ether three times to ensure complete extraction of chlorophylls. The petroleum ether extract (chlorophylls) was made to 25 ml and acetone extract (chlorophyllide) (II) to 10 ml (final concentration of acetone

was about 60%). The absorbances were measured to determine the amounts of chlorophyllides and chlorophylls.

c. Chlorophyllase activity

Chlorophyllase activity was determined in two ways, (1) by measuring the decrease in chlorophyll content and (2) by estimating the conversion of chlorophylls to chlorophyllides during incubation.

The chlorophyll content in the petroleum ether extract after separation was determined spectrophotometrically by the formulae developed by Vernon (1960). The chlorophyll content in buffer solution without the enzymes (or in boiled enzyme solution) was also measured as a control after the usual separation. By comparing these two results, the chlorophyllase activity was calculated as the decrease of the chlorophyll content in the tube containing the enzyme extract.

The second method for measurement of chlorophyllase activity was as follows: To the test tube (I) (above), 21 ml of 100% cold acetone was added and shaken well (the chlorophylls and chlorophyllides would go into the solution as a homogenous mixture). The mixture was centrifuged at 8000 rpm for 10 minutes to remove precipitated proteins. Chlorophylls and chlorophyllides in the supernatant layer were determined spectrophotometrically at 660 nm according to the method of Holden (1961) and Bacon and Holden (1970) based on the fact that chlorophyll and chlorophyllides have the same adsorption spectrum. The percentage of conversion of chlorophylls into chlorophyllides was determined by the absorbances (a) of separated chlorophyllides (II), above, and the absorbances (b) of combined chlorophylls and chlorophyllides at 660 nm. The chlorophyllase activity, as percentage of conversion

of chlorophylls to chlorophyllides, was equal to (a) x 100 divided by (b) x 2.5 (dilution factor).

5. Protein determination

The proteins from spinach leaves were extracted according to Klyne (1974). The proteins in this extract as well as in the crude enzyme (chlorophyllase) extract were determined according to Lowry *et al.* (1951).

Test tubes containing 0, 0.4, 0.8, 1.2 and 1.6 ml of the standard solution (0.1% bovine serum albumin) and suitable quantities of the samples (0.1-0.2 ml) were brought to 1.0 ml with 1.0 N NaOH solution.

Five ml of copper sulfate-sodium potassium tartrate and Na_2CO_3 solution (50 ml of solution A (2% Na_2CO_3) and 1.0 ml solution B (1% $CuSO_4$ solution mixed with an equal volume of a 2% sodium potassium tartrate solution, mixed immediately before use) were added to each of the test tubes. The test tubes were shaken and then allowed to stand for ten minutes at room temperature. Phenol (Folin-Ciocalteau) reagent (0.5 ml 1 N) was then added to each of the test tubes with instantaneous and vigorous mixing. The absorbances were read after thirty minutes and plotted against the protein concentrations. The protein concentration in the extract was then determined from this standard curve.

C. Physiological Experiments

1. Ethylene treatment

(i) Treatment of different plant species with 100 ppm ethylene

In the search for the most suitable material for further

experiments, three types of plants were assayed, namely *Chlorella prenoidosa* L., a unicellular algae, *Lemna minor* L., a small aquatic plant, and spinach (*Spinacea oleracea* L.) leaves.

Ethylene, in compressed air, was chosen to treat spinach leaves, *Lemna* and *Chlorella* cultures under continuous flow at the rates of 200 ml/min, 50 ml/min and 200 ml/min respectively. The difference in the rate flow rate was imposed by the different sizes of the containers used.

Spinach leaves with petioles were picked immediately before the experiment from spinach (whole plants) that were purchased from the Edmonton City Market (one day after harvest). The intact leaves were washed with tap water first and later with deionized double distilled water and dried carefully with Kleenex tissues. The drying process was necessary to prevent fungal growth during the treatment. Immediately after drying, four or five leaves of different sizes (total weight approximately ten grams) were placed in 60 ml deionized double distilled water in a beaker which was placed in a jar equipped with air inlet and outlet units, and left on the flow bench during the duration of the treatment. Compressed air (control) or ethylene in compressed air, both were free of CO_2 by passing through a U-tube containing drierite and Lithasorb (indicating grade of anhydrous lithium hydroxide) and then passed continuously through the jar containing the samples. Other treatment conditions were as follows: light intensity, 50 ft candles; room temperature 22 ± 1°C. For *Chlorella* and *Lemna*, the treatment conditions were similar to their growth conditions except that compressed air (control) or 100 ppm ethylene balanced with compressed air was passed through the cultures. During treatment, in the case of

Lemna, the flask was fitted with a white rubber stopper equipped with an air inlet and air outlet, and these inlets and outlets were guarded with de-oiled cotton.

(ii) Treatment of detached spinach leaves

As can be inferred from the Results (I.A.I.), detached spinach leaves were selected for further studies because of quick and pronounced effect of ethylene on the degradation of chlorophyll. Once the material, *i.e.* detached spinach leaves, was chosen, the following method of treatment had to be worked out.

a. Choice of ethylene concentration

Three concentrations of ethylene in air, namely 20, 50 and 100 ppm were tried for optimal efficiency.

b. Experimental procedures

For treatment, the leaves from spinach grown in growth chamber or other sources where specified, were as described before (see Methods C.1.(i)) placed in a jar equipped with inlet and outlet units with their petioles dipped into a beaker containing 60 ml of distilled water. For the experiments with cytokinins, inhibitors of protein synthesis, solutions of these chemicals were placed in the beaker instead of water. Compressed air or ethylene balanced with compressed air was passed continuously (unless indicated otherwise) through the jar containing the sample. Carbon dioxide (in the air stream or ethylene mixture) was removed by passing through a U-tube containing drierite and Lithasorb. The flow rate of air stream with or without ethylene was 200 ml per minute. The other conditions of the treatment were a light intensity of 50 ft candle (unless otherwise indicated) and a temperature of 22 ± 1°C.

The combined effect of CO_2 and ethylene on the degradation of

chlorophyll was studied next. A similar set-up was prepared with spinach leaves, and different concentrations of CO_2 plus 50 ppm of ethylene (balanced with compressed air) were introduced into the jar containing the sample (the concentrations of CO_2 were 80 ppm, 1%, 5%, 10%, 25%, and 50%). The corresponding concentrations of CO_2 (balanced with air), without the addition of 50 ppm ethylene, were used as a control.

2. Photosynthetic studies

(i) Measurement of respiration and photosynthesis by infra-red gas analyzer

a. Equipment

The CO_2 assimilation (photosynthesis) and evolution (respiration) of spinach leaves were measured with a Beckman Infra-red Gas Analyzer, model 215 (IRGA).

An open gas-exchange system was used (Šesták and Čatský, 1971) in which the incoming air stream (containing 338 ppm CO_2) was divided, one portion going through the enclosure containing a plant and the other going directly to the analyzer (Fig. 3a). The enclosure consisted of two pieces of plexiglass to form a chamber (2.5 x 15 x 25 cm) (Fig. 3b). Terostat type 7 gum (Teroson-Werke D6900, Heidelberg 1, P.O. Box 1720, West Germany) and screws were used to prevent leakage from the chamber after the spinach leaf was placed in the chamber. The petiole stuck out and dipped in deionized double distilled water to prevent wilting during the experiment.

The sample chamber of the IRGA and other components of the system for gas mixing was inter-connected by means of polyvinyl chloride tubing (6 mm I.D.). The air stream (or air containing 50 ppm ethylene)

Fig. 3. An open gas exchange system for the measurement of net CO_2 assimilation and respiration.

a. The gases mixing system

F : glass wool filter

X : needle valve for controlling gas flow

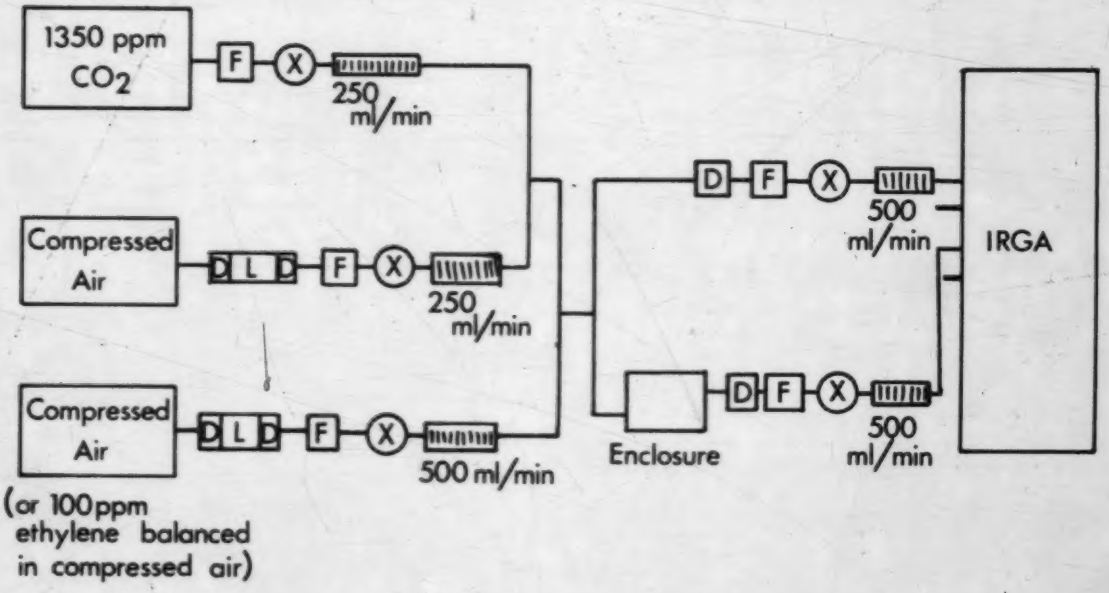
D : Drierite for removing moisture

L : Lithasorb to absorb CO_2 without absorbing ethylene

IRGA : Infra-Red Gas Analyser

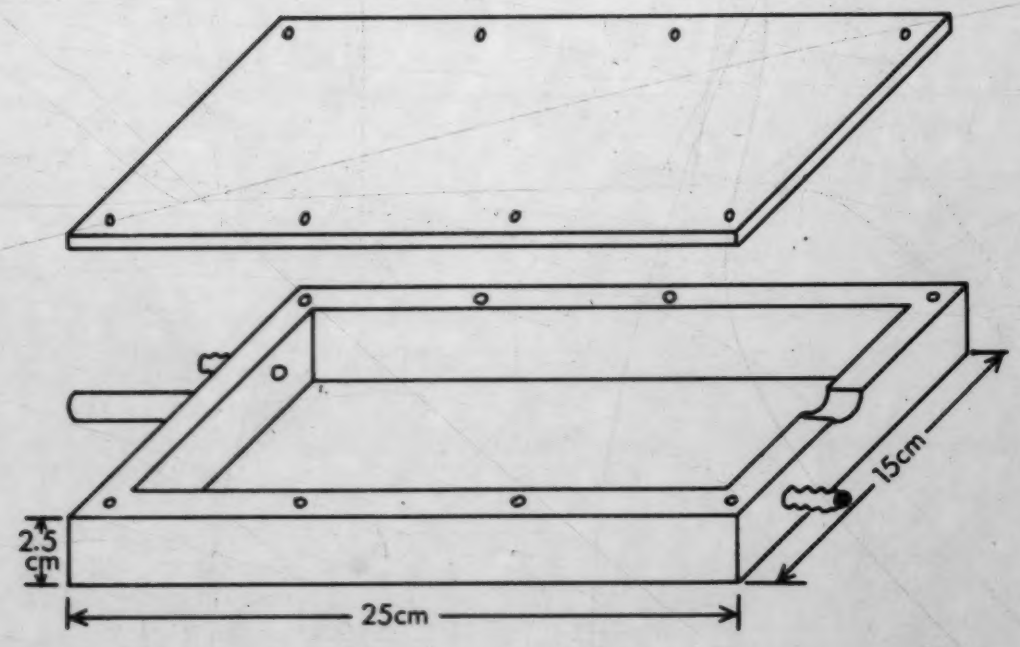
b. The enclosure. The enclosure consisted of two pieces of plexiglass to form a chamber. Terostat type 7 gum and screws were used to prevent leakage from the chamber after the spinach leaf was placed in the chamber. The petiole sticking out through an opening at the side of the chamber. The enclosure also provided an inlet and an outlet for the gassing system to gas through as shown in Fig. 3a.

a



b

THE ENCLOSURE



was controlled by NUPRO needle valves and Matheson flow meters (No. 601 and 602). Each flow chart of the flow meter was recalibrated with a soap bubble gas flow meter under the experimental conditions.

The IRGA was calibrated to detect a small CO_2 differential (31.7 ppm), with a reference base considerably above zero (about 300 ppm). To do this the reference and measuring cells of the IRGA were flushed with two calibration gases with CO_2 concentrations at/or near the limits of the desired differential. The calibration of standard gases was done by Dr. J. Mayo, Department of Botany, University of Alberta by the method of Bate *et al.* (1969).

Light for the illumination of the leaves was provided by a reflector spot incandescent lamp (Sylvania, 150 watts). The light intensity, as measured with an illuminating meter (Weston Electrical Instrument Co., model 756), was 2500 ft candles. The considerable amount of heat produced by the incandescent lamp was absorbed by water circulated through two culture bottles. The light quality for photosynthesis was not affected by this arrangement. The spectra of the incandescent light (without and with water filters) are shown in Fig. 4.

b. Measurement of CO_2 assimilation and dark respiration of spinach leaves

In IRGA the measurement of CO_2 is based on a concentration differential between the reference and the sample cell. When the CO_2 concentration in the set-up line of a sample cell containing spinach leaves increased as a result of respiration, the detector registered an increase on the recorder chart. If the concentration in sample cell decreased because of CO_2 assimilation, the detector registered a

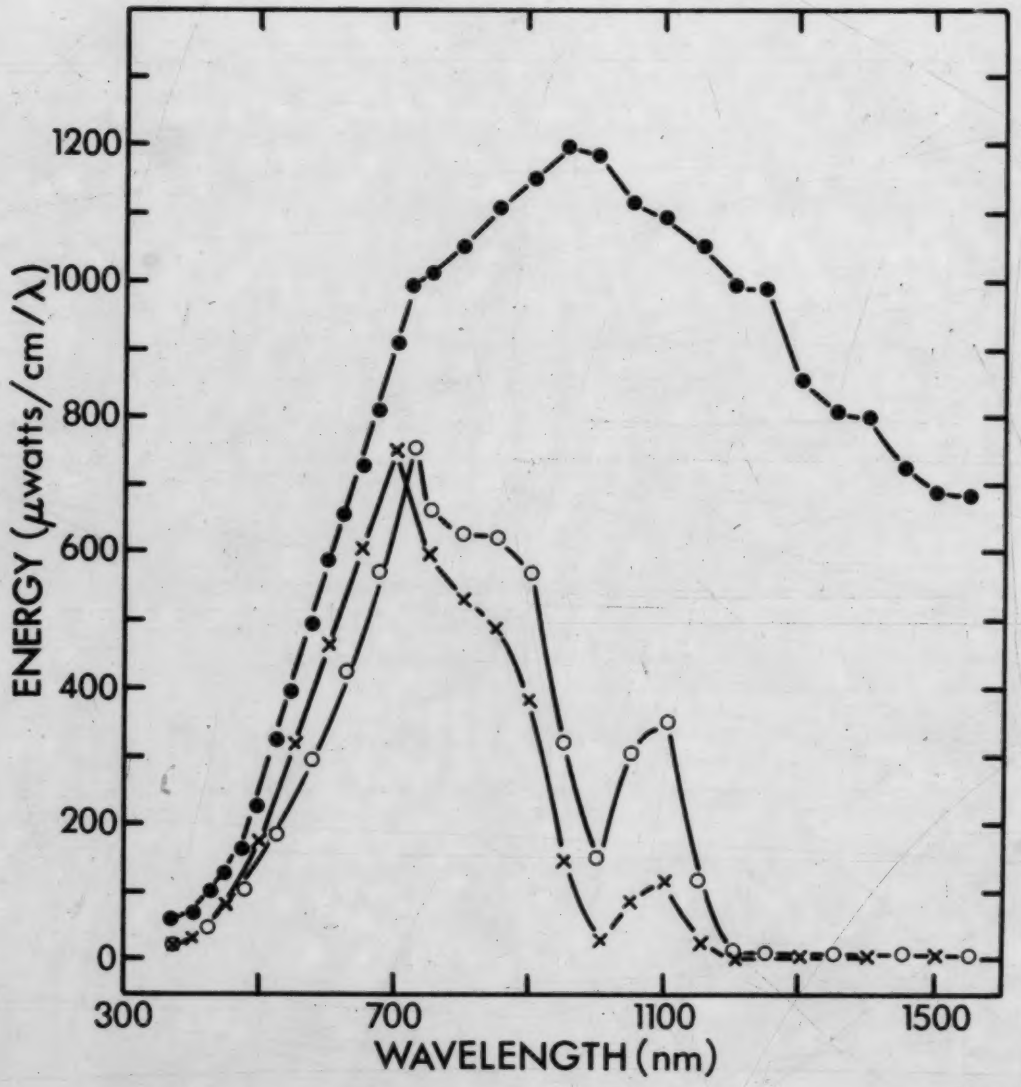


Fig. 4. The spectra of light from incandescent Sylvania 150 watt reflector spot lamp, with and without water filters.

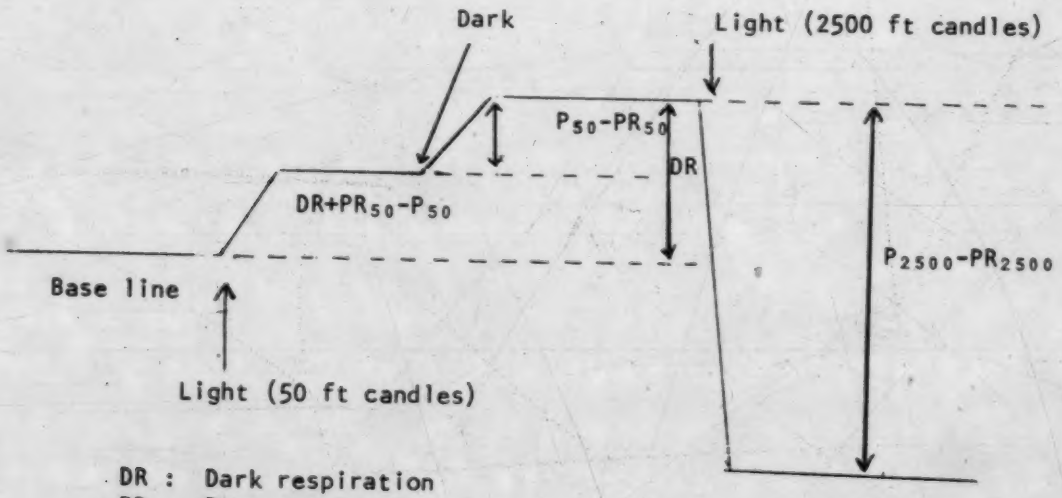
● —— ● Spectrum of light from incandescent lamp

x —— x Spectrum of light from incandescent lamp with water jacket of oxygen monitor as filter

o —— o Spectrum of light from incandescent lamp with culture bottle of water as filter

The light energy was measured by Spectroradiometer Model SR, Instrumentation Specialities Company Inc., Lincoln, Nebraska, U.S.A.

decrease on the recorder chart. The base line is obtained when both reference and sample cell contained the same concentration of CO_2 : 338 ppm, and from the differences between base line and the recorded shifts one obtains the percent decrease or increase in CO_2 concentration. By using the conversion factor, as described below, the amount of CO_2 assimilation or evolution by spinach leaves was calculated. A typical trace from an experiment is shown below.



DR : Dark respiration
PR : Photorespiration
P : Photosynthesis

When CO_2 concentration was calculated as µmoles CO_2, the flow rate had to be known and precisely measured and controlled by needle valve and flow meter. The flow rate of 500 ml/min was selected because the flow rate must be large enough to maintain a certain CO_2 concentration in the sample chamber, but the flow rate should not be too large either for IRGA to measure accurately. The calculation of CO_2 concentration as µmoles is as follows:

Flow rate for the experiment = 500 ml/min or 30 l/h

Standard gases differential, 31.7 ppm ≅ 15.4% on recorder chart

Conversion factor = 30 x 31.7 µl/26.53 µl x 15.4 =

2.328 µmoles CO_2/h

(1 µmole CO_2 = 26.53 µl at Edmonton, 30 l of 31.7 ppm CO_2 should contain 30 x 31.7 µl CO_2)

Therefore, net CO_2 assimilation under 2500 ft candles

= $(P_{2500} - PR_{2500})$ x 2.328 µmoles CO_2/h

Net CO_2 assimilation under 50 ft candles

= $(P_{50} - PR_{50})$ x 2.328 µmoles CO_2/h

Dark respiration = DR x 2.328 µmoles CO_2/h

Results of CO_2 assimilation of spinach leaves were based on chlorophyll weight and leaf area. Determination of chlorophyll was outlined in a previous section. The measurement of leaf area was as follows: a spinach leaf was spread well on a white paper and the outline of the leaf was drawn. This paper was then cut along the outline and was passed through a Leaf Area Meter (Harachi) where the total leaf area was measured automatically and the dimensions were given in square decimeters.

(ii) Photosynthetic studies of isolated chloroplasts

a. Isolation of chloroplasts

Intact chloroplasts were prepared from spinach leaves purchased from the local market or grown in the greenhouse or university farm. The isolation method was similar to that of Jensen and Bassham (1966) except that sodium ascorbate was not added to the isolation medium.

The morphology of isolated chloroplasts particularly the relationship of intactness of the outer envelope and the CO_2 fixation is believed to affect the efficiency of CO_2 fixation and O_2 evolution.

Therefore, different methods of homogenization were tested to determine their effect on the morphology of the chloroplasts. A meat grinder, a mortar and pestle and a semi-micro vessel Waring blendor were tried. A Waring blendor gave the least breakage of chloroplasts and the lowest contamination as seen under light microscope (Leitz, Germany). A homogenization time of five seconds was found to be optimal; longer periods caused considerably larger amounts of breakage while shorter periods yielded insufficient amounts of chloroplasts. Accordingly, the preparation method was as follows: About 15 g of leaves were washed, dried, chilled and the mid ribs were removed. The leaves were then torn into small pieces by hand and subsequently placed in a semimicro homogenizing vessel and a Waring blendor and blended for five seconds at low speed in 25 ml of cold MES buffer (pH 6.1). The homogenate was filtered through eight layers of cheesecloth and centrifuged at 5000 rpm for 50 seconds. The pellet was suspended in approximately 1.5 ml of HEPES buffer, pH 7.5, and kept at 0°C for further O_2 evolution and CO_2 fixation experiments.

The components of buffers used for isolation, suspension and reaction were as follows:

Master buffer (A): $NaNO_3$ (2 mM), $MnCl_2$ (1 mM), $MgCl_2$ (1 mM), K_2HPO_4 (0.5 mM), EDTA (2 mM), sorbitol (0.33 M).

Isolation (MES) buffer: A + MES (50 mM) + NaCl (20 mM), pH 6.1.

Suspension (HEPES) buffer: A + HEPES (50 mM) + NaCl (20 mM), pH 6.7.

Reaction (TRICINE) buffer: A + TRICINE (50 mM) + $Na_4P_2O_7$ (5 mM), pH 8.1.

The isolated chloroplasts were also purified by zonal sucrose gradient centrifugation by the method of Hamman (1973).

b. Measurement of oxygen evolution

The oxygen evolution of isolated chloroplasts from spinach leaves was measured at 20°C in oxygen monitor (Yellow Springs Instrument Co., Model 53) equipped with a Clarke-type electrode inserted into Lucite reaction vessel containing the reaction mixture. Prior to transfer into the vessel, the reaction mixture was bubbled with purified nitrogen to remove most of the dissolved oxygen. When the oxygen evolution experiment was done in an ethylene-air atmosphere, 50 ppm of ethylene in nitrogen with a flow rate of 50 ml/min was bubbled through 100 ml reaction mixture (TRICINE buffer) for 80 minutes at 20°C. This was the time required for stabilizing the concentration of ethylene in the reaction mixture (Fig. 5).

Usually, 0.4 ml of HEPES buffer suspension containing intact chloroplasts was added to 4.6 ml of reaction mixture. This total reaction mixture contained 15 µmoles of Na_2CO_3 (CO_2 source) or 14 µmoles of potassium ferricyanide as electron acceptor for measuring the Hill reaction (O_2 evolution). The light source was a reflector spot incandescent lamp (Sylvania) and the intensity was 2500 ft candles (measured with a illuminating meter; Weston Electrical Instrument Co., model 756). It is important to note that the light quality for photosynthesis was not affected by passing through the water jacket of the oxygen monitor (see Fig. 4). A full scale deflection of the oxygen monitor equalled a change in O_2 concentration of 6.92 µmoles.

c. Determination of CO_2 fixation

The rate of CO_2 fixation was measured by incubation of the

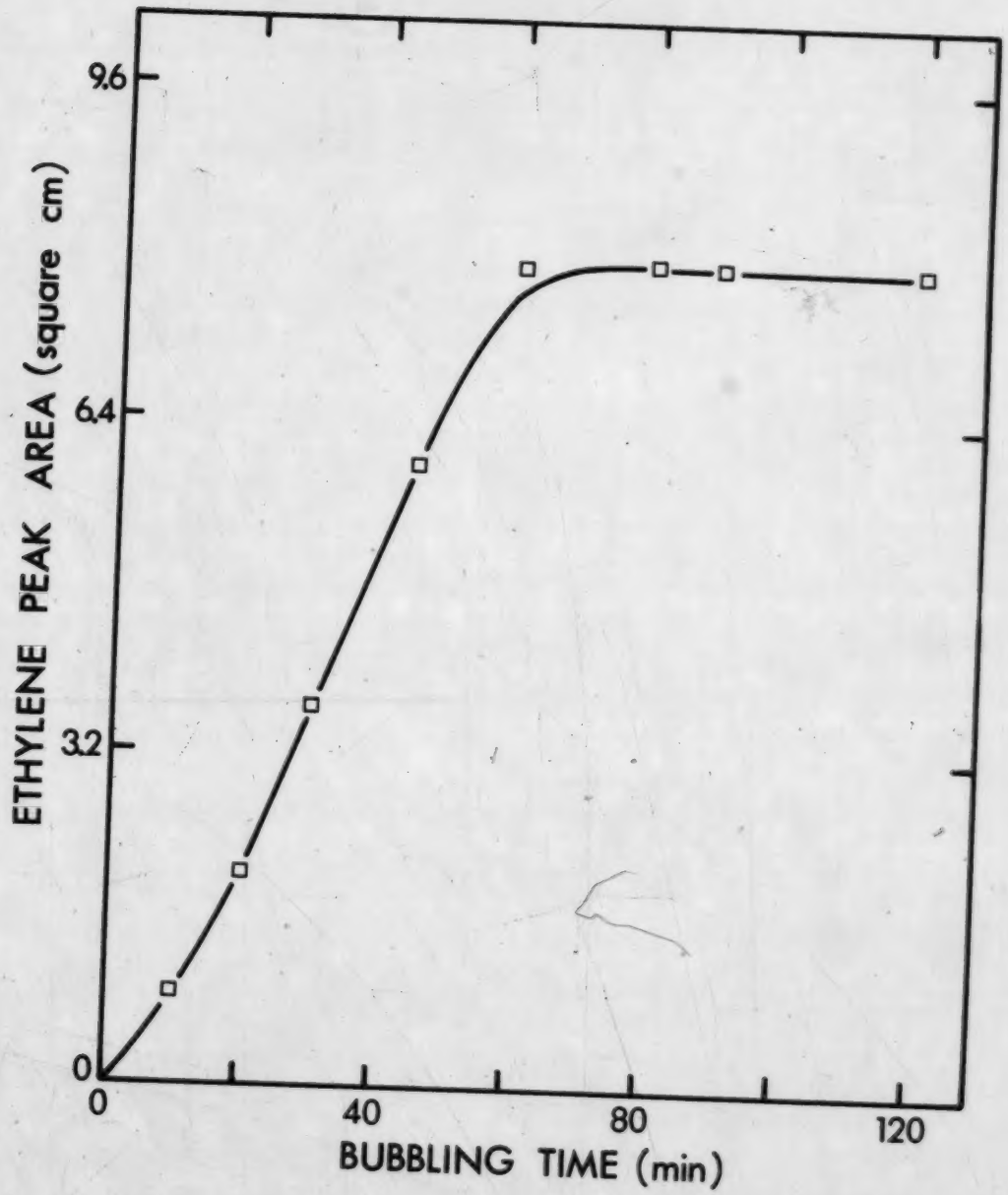


Fig. 5. Time required for the stabilization of ethylene dissolved in TRICINE buffer at 20°C.

50 ppm ethylene in nitrogen, with a flow rate of 50 ml/min was bubbled through 100 ml TRICINE buffer at 20°C for various lengths of time. An aliquot (20 ml) of each treated buffer solution containing ethylene was transferred to a gasing system. Ethylene was collected and measured by gas chromatography.

chloroplasts with Na_2CO_3 in a reaction vessel of the oxygen monitor. The reaction mixture for measurement of the CO_2 fixation usually contained 15 µmoles of Na_2CO_3 containing approximately 10 µc of ^{14}C. The reaction was terminated by adding 20% trichloroacetic acid (TCA weight/volume). An aliquot of 0.2 ml reaction mixture was added to a scintillation vial containing 0.2 ml of 20% TCA and then warmed to 50°C for five minutes to decompose unreacted carbonate (Schacter *et al.*, 1971). Then, 15 ml of Aquasol fluor (a New England Nuclear Co. premixed scintillator) was added to each vial and the samples counted in a scintillation counter (Nuclear Chicago, model Unilux II).

(iii) Electron microscopic studies of spinach leaves and of their isolated chloroplasts

a. Spinach leaves

Fresh spinach leaf slices (width less than 1 mm) were fixed in a mixture of 3% glutaraldehyde plus 3% formaldehyde in 0.1 M phosphate buffer (pH 7) overnight at 4°C. The specimens were then placed in phosphate buffer at 22°C for four hours. Tissues were then rinsed with the phosphate buffer and then dehydrated with a graded ethanol series (50, 60, 75, 85, and 95% v/v ethanol in water), and then rinsed two times with propylene oxide. The tissues were then transferred to 1:1 (v/v) mixture of propylene oxide and araldite for 24 hours prior to embedding in pure araldite. Polymerization was performed at 60°C for 36-48 hours. Sectioning was done on a Sorvall ultramicrotome provided with a glass knife. Silver and gold-tint sections were picked up on copper grids (100 mesh size) coated with Formvar and stained with 0.2% uranyl acetate for two hours, followed by 0.2% lead citrate for two minutes. The fixed tissues were examined with a Phillips, model 200

electron microscope (E.M.).

b. Isolated chloroplasts

The pellet of isolated chloroplasts was fixed overnight at 4°C in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 and then centrifuged at 3020 g for ten minutes. The fixed chloroplasts were washed without agitation three times, each for thirty minutes, in phosphate buffer at 4°C. Each washing step was followed by a centrifugation at for ten minutes to recover chloroplasts. Then the samples were post-fixed with 2% OsO_4 in phosphate buffer for two hours at 4°C and washed with phosphate buffer three times at 4°C for a total time of one hour. The samples were then dehydrated at 4°C with a series of ethanol-water solutions (20, 40, 60, 80, and 95% (v/v)). Two hour intervals were maintained between stages. Infiltration of the chloroplast into the Spurr's resin was done drop by drop overnight and the impregnated Spurr's resin was allowed to harden for thirty-six hours at 60°C. The rest of the procedure, sectioning, staining and E.M. examination was the same as for spinach leaves.

RESULTS

A. Effects of Ethylene on Chlorophyll Degradation

1. Chlorophyll degradation in different plants

The effects of 100 ppm ethylene in air on chlorophyll degradation in different plants are shown in Table 1. Among the three plants tested, the effect of ethylene on chlorophyll degradation was highest in detached spinach leaves (later mentioned as spinach leaves), with only 45% of total chlorophylls compared to that of the air control. The ratio of Chl a/Chl b was not markedly affected by ethylene.

Ethylene-induced degreening of spinach leaves appeared as an initial yellowing of the leaf tip which gradually spread downward towards the petiole. Although yellowing of *Lemna* fronds was less rapid than for spinach, it was more homogenous. It must be remembered that *Lemna* fronds are small and whole leaf in contact with the culture medium, so that the dissolved ethylene is evenly dispersed. It might be of interest to note that the roots which were hanging in normal *Lemna* cultures, detached easily in ethylene treated samples. This may be related to the abcission of leaves and fruits induced by ethylene. Ethylene (10, 20, 50, 100, and 250 ppm) did not have any effect on photosynthesis, namely O_2 evolution and CO_2 fixation of *Chlorella*. Ethylene did not decrease in chlorophyll content of *Chlorella* either. However, ethylene did increase in respiration of *Chlorella*.

2. Effects of ethylene on chlorophyll degradation in spinach leaves

(1) Ethylene production by spinach leaves

Spinach leaves produced ethylene. This was demonstrated both in sealed jars and in continuous air stream operation. In continuous air

TABLE 1. The effects on chlorophyll contents of exposure of different plants to a continuous flow of 100 ppm ethylene for six days

Plants	Chlorophyll Content (mg/g dry wt)		% Ethylene treated
	Air treated	Ethylene treated	Air treated X100
Spinach leaf			
Chl a	5.37	2.36	
Chl b	3.41	1.57	
Total Chl	8.78	3.93	45
Chl a/Chl b	1.58	1.50	
Lemna			
Chl a	10.24	7.52	
Chl b	4.90	3.42	
Total Chl	15.14	10.94	72
Chl a/Chl b	2.09	2.20	
Chlorella			
Chl a	13.45	14.26	
Chl b	7.43	7.93	
Total Chl	20.88	22.19	106
Chl a/Chl b	1.81	1.80	

Chlorophyll was extracted and estimated as described under Materials and Methods (B. 2). The data on this table are representative of several experiments and the variation among the experiments was less than ±5% of chlorophyll content.

flow (200 ml/min) 10 nl C_2H_4/g fresh wt/h of ethylene was evolved within the first 18 hours after which it declined to about 0.9 nl C_2H_4/g fresh wt/h by the third day (Table 2). In sealed jars as well, spinach leaves produced large amounts of ethylene (Table 3). The leaves kept in air produced and accumulated relatively large amounts of ethylene until Day 2, after which the level of ethylene in the jar remained more or less constant. The CO_2 concentration in the "air" jar gradually increased up to 1.18% at Day 4. The spinach leaves in the "ethylene" jars produced approximately the same amount of CO_2 in four days.

Another interesting point in this experiment is that spinach leaves seem to take up ethylene. When 20 ppm ethylene was introduced into the atmosphere of the sealed jar containing 10 g of spinach leaves (it was termed as jar 2 in Table 3), only 2 ppm (10% of the original ethylene concentration) was found after four days. One possible explanation for the disappearance of ethylene is that it might have been converted to ethane (Ghooprasert, 1971) by the leaves. Indeed, the amounts of ethane detected by gas chromatography increased with time. However, increases in the amounts of ethane were much less than the decreases in the amounts of ethylene.

(ii) Effects of different concentrations of ethylene on chlorophyll degradation

Concentrations of 20, 50 and 100 ppm of ethylene in air were used for the treatment of spinach leaves. The continuous flow procedure was used. The chlorophyll contents of the different samples expressed as percentages of air controls are shown in Fig. 6. The effect of ethylene on chlorophyll degradation increased with increasing ethylene concentrations from 20 ppm to 100 ppm during the four days of

TABLE 2. Production of ethylene from detached spinach leaves

Collection time (h)	Ethylene evolved (nl/g/h)
0	10.0
1.5	4.8
3.0	3.3
18	1.4
24	1.4
48	1.6
72	0.9

Ethylene was collected for 20 minutes at each of the above times. Compressed air for flushing out ethylene was previously treated by passing through a trap made of mercuric perchlorate (see Materials and Methods, B. 1) to remove existing ethylene in compressed air.

TABLE 3. Changes in ethylene and carbon dioxide concentration in sealed jars containing ten grams of detached spinach leaves

	Jar 1*		Jar 2**	
Time (days)	Ethylene (ppm)	CO_2 (%)	Ethylene (ppm)	CO_2 (%)
0	0	0	20.00	0
1	0.25	0.36	8.78	0.36
2	0.65	0.66	4.28	0.61
3	0.44	0.78	3.38	0.67
4	0.52	1.18	2.02	1.39

Spinach leaves in 1.7l sealed jars were flushed with either compressed air or 20 ppm ethylene in air for one hour before the jars were sealed. Compressed air was freed of ethylene and CO_2 by passing through a trap made of mercuric perchlorate and through a trap made of Drierite and Lithasorb (see Materials and Methods B.1). A stream of 20 ppm ethylene in air only passed through a trap of Drierite and Lithasorb to remove-CO_2.

* Contained air without ethylene.

** Contained 20 ppm ethylene in air.

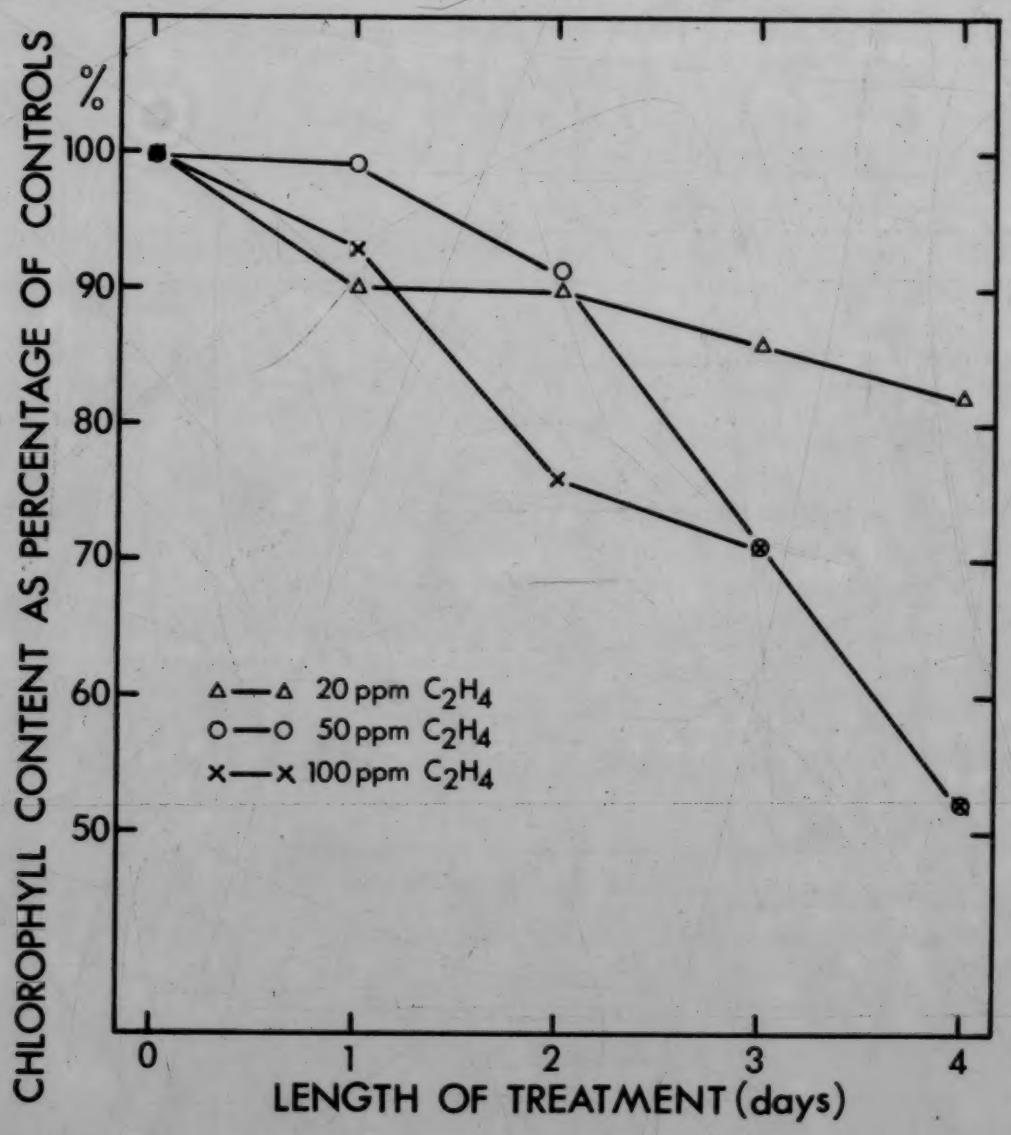


Fig. 6. The effects of different concentrations of ethylene on the chlorophyll content of spinach leaves.

Spinach leaves from whole plants were purchased from the Edmonton City Market, were treated in a continuous flow and with a light intensity of 50 ft candles. The results on this figure are representative of several experiments and the variation among the experiments was minimal.

treatment. In the 20 ppm ethylene treatment, chlorophyll degradation was most pronounced during the first day. Although the chlorophyll content fell in the subsequent days this decrease was gradual. After three days, there was no difference in the chlorophyll content between the samples treated with 50 ppm and 100 ppm ethylene. In view of these data, the concentration of 50 ppm ethylene was chosen for subsequent treatment of spinach leaves.

(iii) Effects of ethylene on the degradation of chlorophyll under different light intensities

Spinach leaves were treated for three days with 50 ppm ethylene to determine its effect on the chlorophyll content of spinach leaves under different light intensities of 50, 500, 2500 and 5000 ft candles. Figure 7 shows that the higher the light intensity, the greater the chlorophyll decrease. The chlorophyll content (as percentage of air control) of spinach leaves under an illumination of 5000 ft candles and 50 ppm ethylene in a continuous gas flow is only 27% while that of a similar sample in the dark was 64%. Light has been shown to have a deleterious effect on chlorophyll (photodestruction). Our data seemed to show that this effect is enhanced by the presence of added ethylene, or *vice versa*. As far as we know, this combined effect has not been reported in the literature.

On the basis of these results, subsequent experiments were carried out under "room" light (50 ft candles) without either extra illumination or special darkening.

(iv) Effects of ethylene on soluble proteins and chlorophyllase activity

Spinach leaves were treated with 50 ppm ethylene and then

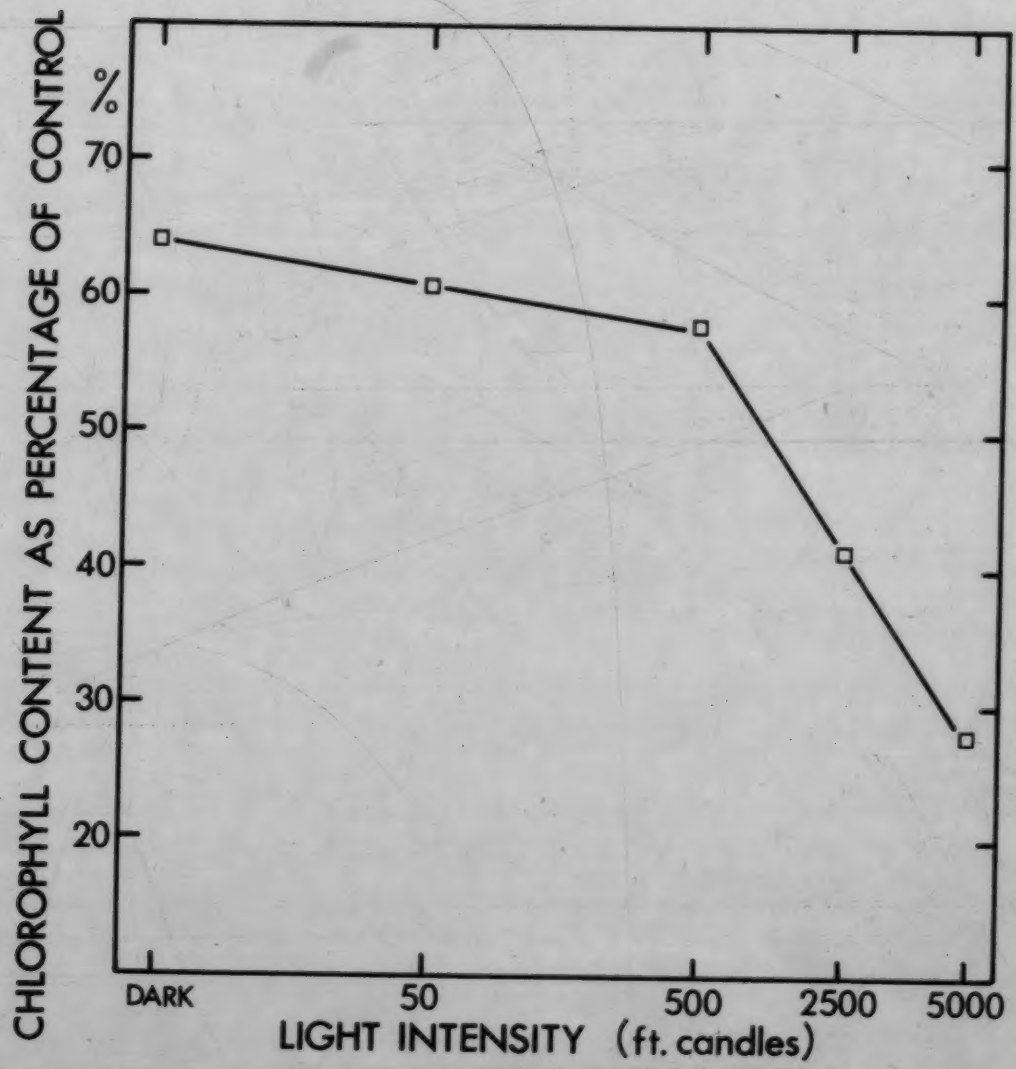


Fig. 7. The effect of 50 ppm ethylene on chlorophyll degradation in detached spinach leaves under different light intensities in continuous gas flow for three days.

soluble protein contents were determined up to Day 4. The results are shown in Fig. 8. The protein contents of ethylene-treated samples increased considerably up to Day 1 and then declined gradually. There was only a slight increase in soluble protein content of air-treated samples up to Day 1 after which it remained constant. The results represented were the average of three experiments.

DNA and RNA content were also determined up to four days. DNA content of ethylene-treated samples was higher at Hour 6, 12 and Day 1 than air control and then decreased with time. The content of RNA increased up to Day 3 compared to air control and then decreased.

It was found that the chlorophyllase activity of ethylene-treated samples in a continuous gas flow increased as compared to air controls during the four days of treatment (Fig. 9). The activity of chlorophyllase, both in ethylene- and air-treated samples, increased with time until Day 3 and then decreased. The interesting point is that the increase in activity was followed by the decrease in chlorophyll content in both cases except on Day 4.

In the case of spinach leaves treated with 20 ppm ethylene in the sealed jars, the activity of chlorophyllase was slightly lower than that of the air control after two days of treatment (Fig. 10). The chlorophyll content of Day 2 and Day 3 ethylene-treated samples was slightly higher than in the air controls. These different effects from that in continuous gas flow could be because of CO_2 accumulation in the sealed jars. The effect of high ethylene concentration (100 ppm) and longer treatment (six days) on chlorophyll degradation of detached spinach leaves in sealed jars was also studied. The results are shown in Table 4. Ethylene effect was reduced in the sealed jars as compared

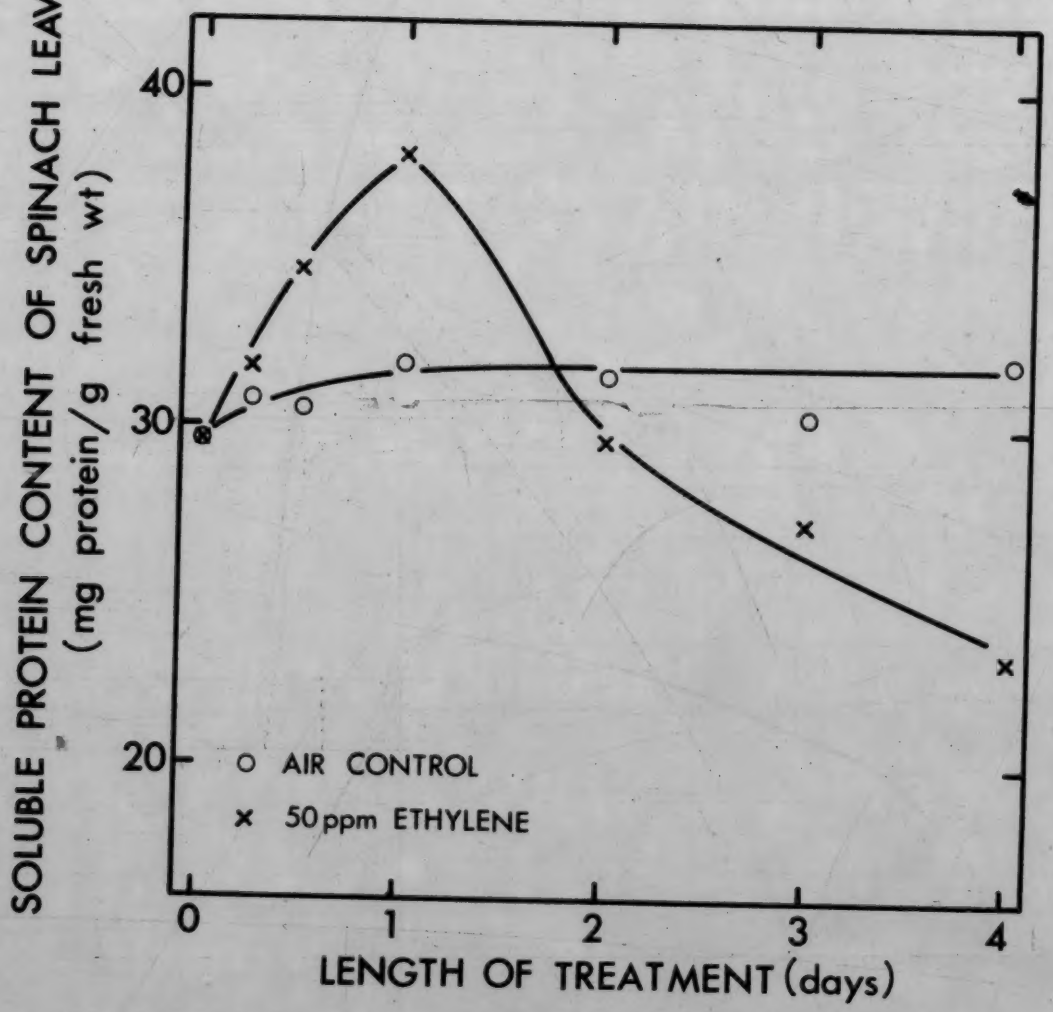


Fig. 8. The effects of 50 ppm ethylene on soluble protein content of spinach leaves.

The results on this figure were representative of several experiments and the variation among the experiments was minimal.

Fig. 9. The effect of 50 ppm ethylene on chlorophyllase activity and chlorophyll degradation in spinach leaves in continuous gas flow.

Legend: o air control

x ethylene

Broken line ----- Chlorophyll content

Solid line ——— Chlorophyllase activity

The chlorophyllase activity was assayed as described under Materials and Methods (B. 4). The results on this figure were the average of duplicate experiments and the variation of the experiments was minimal.

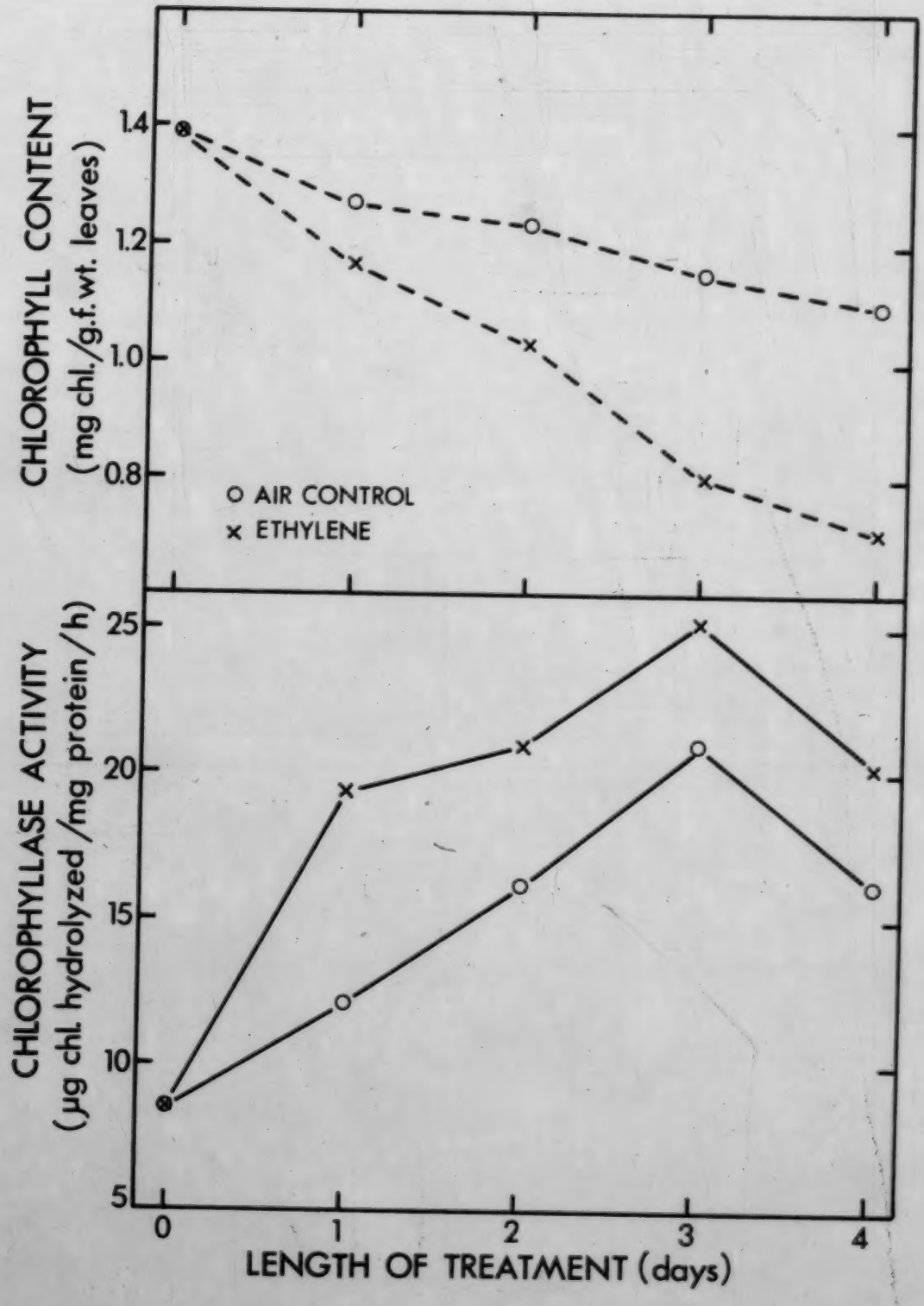

CHLOROPHYLL CONTENT
(mg chl./g.f.wt. leaves)
1.4
1.2
1.0
0.8
O AIR CONTROL
× ETHYLENE
CHLOROPHYLLASE ACTIVITY
(µg chl. hydrolyzed /mg protein/h)
25
20
15
10
5
0
1
2
3
4
LENGTH OF TREATMENT (days)

Fig. 10. The effect of 20 ppm ethylene on chlorophyllase activity and chlorophyll degradation in spinach leaves in 1.7 litre sealed jars.

Legend: o in air

x in ethylene

Broken line ----- Chlorophyll content

Solid line ——— Chlorophyllase activity

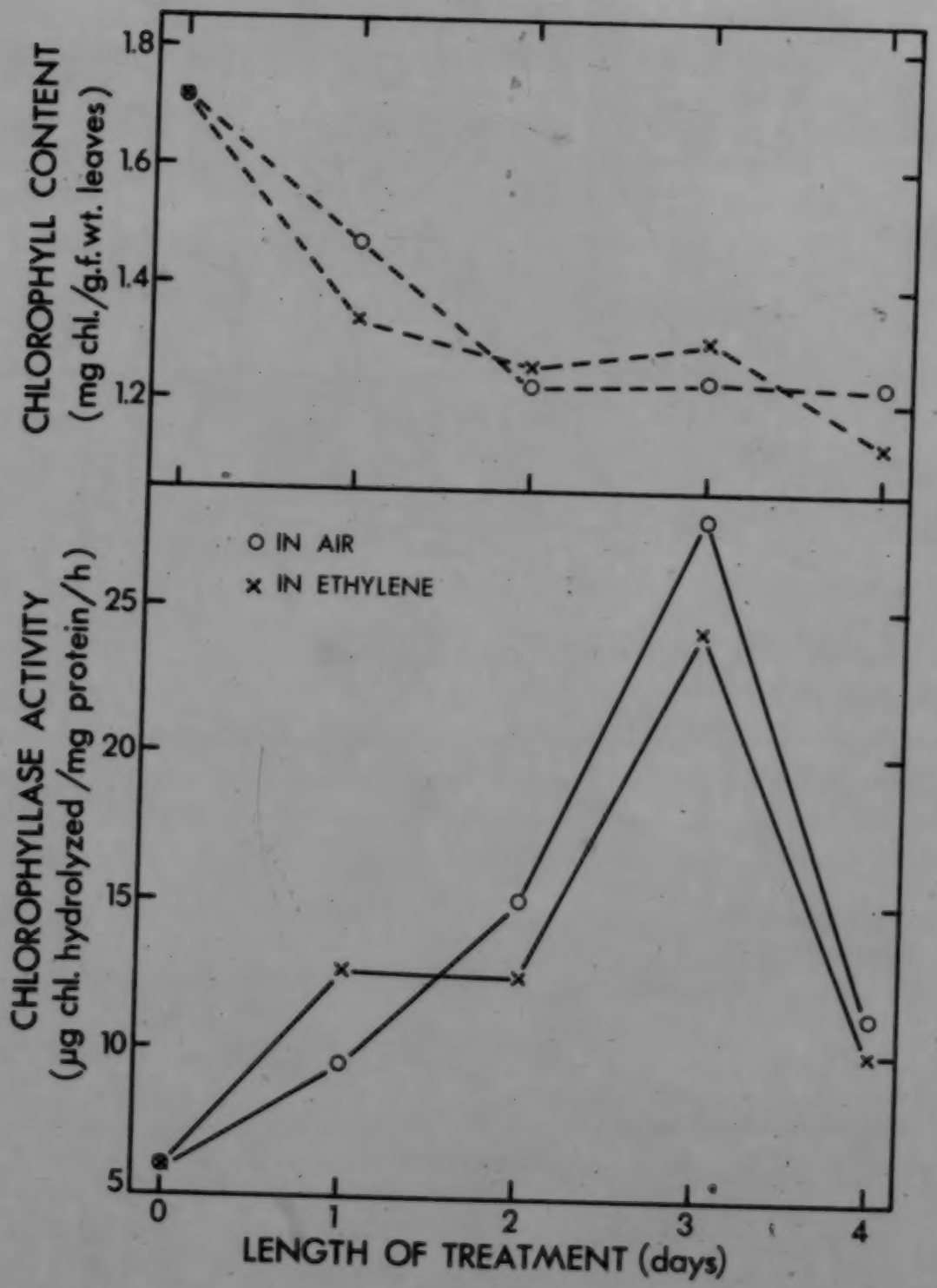

CHLOROPHYLL CONTENT
(mg chl./g.f.wt. leaves)
1.8
1.6
1.4
1.2
CHLOROPHYLLASE ACTIVITY
(µg chl. hydrolyzed /mg protein/h)
O IN AIR
× IN ETHYLENE
25
20
15
10
5
0
1
2
3
4
LENGTH OF TREATMENT (days)

TABLE 4. The effect of 100 ppm ethylene on the degradation of chlorophyll of spinach leaves held in 1.7 l sealed jars for 6 days

	Chlorophyll Content (mg Chl/g dry wt)		
	Air Control	100 ppm Ethylene	Chlorophyll content as percentage of air control
Chl a	6.01	4.12	69
Chl b	3.18	2.38	74
Total Chl	9.19	6.50	71
Chl a/Chl b	1.89	1.73	

Chlorophyll was extracted with 80% acetone and estimated spectrophotometrically at 649 nm and 665 nm for chlorophylls a and b according to the formulae developed by Vernon (1960). The results on this table were the average of duplicate experiments and the variation of two experiments was minimal.

to that of samples in continuous ethylene flow (Table 1).

As can be seen also in Fig. 11, the addition of 1.2% CO_2 to air containing 20 ppm ethylene (in continuous flow) reduced chlorophyllase activity and slightly increased chlorophyll content of spinach leaves compared to those of samples treated with 20 ppm ethylene alone.

(v) The effect of some inhibitors of protein synthesis on ethylene-induced degradation of chlorophyll

In order to find out if ethylene-induced degradation of chlorophyll was mediated by a *de novo* synthesis of proteins, the effects of inhibitors of protein synthesis, namely cycloheximide and chloramphenicol (D-C-7-threo-chloramphenicol) and the chlorophyll content of spinach leaves were studied. The results are recorded in Table 5. Cycloheximide at a concentration of 12 µg/ml completely prevented chlorophyll degradation in the presence of ethylene. Applications of 150 µg and 1.5 mg chloramphenicol/ml resulted in an increase in chlorophyll content as compared to that without chloramphenicol. However, the increase was much lower than that in the case of cycloheximide. This would suggest that the degradative enzymes are synthesized mainly in the cytoplasm, because cycloheximide is known to inhibit protein synthesis occurring in the cytoplasm.

(vi) The effect of 2,4-dinitrophenol (DNP) on ethylene-induced degradation of chlorophyll

The influence of DNP on the protection of chlorophyll against 50 ppm of ethylene treatment is shown in Table 6. It proved to have a protective effect toward chlorophyll. However, this effect decreased with increasing concentrations of DNP (10^{-5}, 10^{-4}, and 10^{-3} M). If the

Fig. 11. The effects of 20 ppm ethylene and 1.2% CO_2 on chlorophyllase activity and chlorophyll degradation of spinach leaves in continuous gas flow.

Legend: o Ethylene (20 ppm) + 1.2% CO_2

x Ethylene (20 ppm) in air (without CO_2)

Broken line ----- Chlorophyll content

Solid line ——— Chlorophyllase activity

The results in this figure were the average of duplicate experiments and the variation of two experiments was minimal.

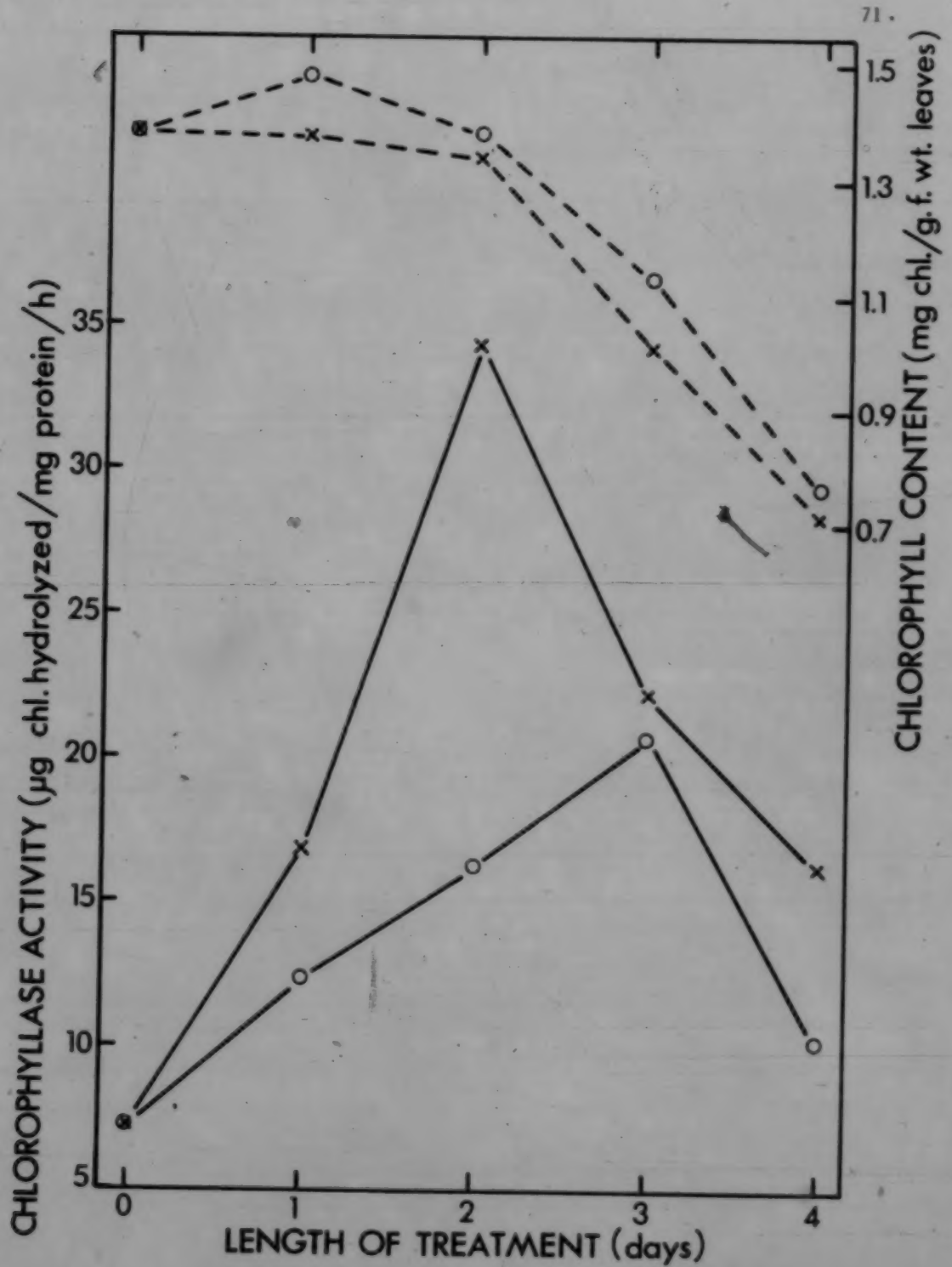

CHLOROPHYLLASE ACTIVITY (µg chl. hydrolyzed/mg protein/h)
CHLOROPHYLL CONTENT (mg chl./g. f. wt. leaves)
LENGTH OF TREATMENT (days)
5
10
15
20
25
30
35
0.7
0.9
1.1
1.3
1.5
0
1
2
3
4

TABLE 5. Effects of some inhibitors of protein synthesis on the protection of chlorophylls of detached spinach leaves against ethylene treatment for three days

Treatments	Chlorophyll content as % of control
Air control	100
50 ppm Ethylene	64
50 ppm Ethylene + cycloheximide, 1.2 µg/ml	67
50 ppm Ethylene + Cycloheximide, 12 µg/ml	101
50 ppm Ethylene + Chloramphenicol, 150 µg/ml	76
50 ppm Ethylene + Chloramphenicol, 1.5 mg/ml	79

Spinach was grown in growth chamber for four weeks. Detached leaves were placed in the solution of cycloheximide or of chloramphenicol and treated with 50 ppm ethylene at a light intensity of 50 ft candles for three days. Ethylene treatment was in a continuous gas flow. The results shown in this table are the average of duplicate experiments and the variation among two experiments was minimal.

TABLE 6. The effects of 2,4-dinitrophenol on the protection of chlorophyll against ethylene treatment for three days

	Chl content (mg chl/g acetone powder)	Chl content as % of air control
Air control	20.64	100
DNP, 10^{-4}M	19.40	94
50 ppm ethylene	13.12	64
DNP, 10^{-5}M	20.32	98
DNP, 10^{-4}M	18.82	91
DNP, 10^{-3}M	14.65	71

chlorophyll content of air control were taken as 100, 50 ppm ethylene treatment without the addition of DNP reduced the chlorophyll content of spinach leaves to 64% and with the addition of increasing concentration of DNP, the chlorophyll contents were 98%, 91% and 71% respectively. Air control with DNP (10^{-4}M) when tested for chlorophyll content gave 94% as compared to air control without DNP. Therefore, the chlorophyll content decreased slightly after the addition of DNP. These results indicate that the critical level of ATP produced by the mitochondria might be needed for protecting the normal structure of chloroplast and also to prevent the degradation of chlorophylls by structural changes of chloroplasts. Three inhibitors of chlorophyll synthesis, i.e., hemin, malonate and maleate were administered to spinach leaves kept at an illumination of 50 ft candles for three days. The results indicate that under these conditions, ethylene stimulated chlorophyll synthesis.

(vii) The effect of ethylene on the products of chlorophyll degradation

Chlorophylls and other pigments of spinach leaves were analyzed by thin layer chromatography (TLC) and Sephadex LH20 gel column chromatography. The results show no qualitative difference between the 50 ppm ethylene-treated samples and air controls (Figs. 12, 13, 14). The only difference between the ethylene-treated samples and air controls when the pigments were separated by LH20 gel column chromatography was in the position of the green-yellow and orange bands above the green chlorophyll band (11). In air controls, the green-yellow band came later while this was reversed in ethylene-treated samples. These

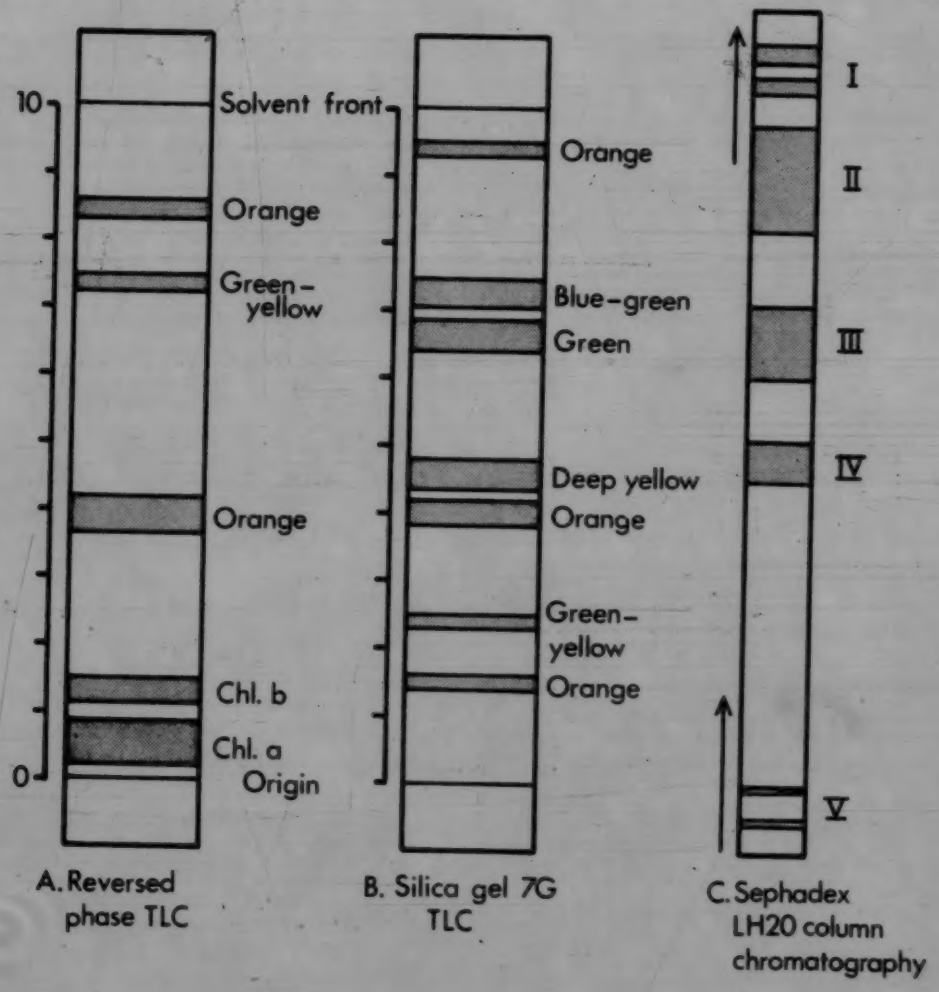


Fig. 12. Diagrammatic representations of thin-layer and Sephadex LH20 gel column chromatographs for separation of pigments from 50 ppm ethylene treated spinach leaves in continuous gas flow. Air controls were not qualitatively different.

Legend:

A. Reversed phase TLC
Layer: Kieselguhr G impregnated with peanut oil in heptane (14%).
Solvent system: Methanol-acetone-water (20:4:3).

B. Silica gel 7G TLC
Layer: 250 μ thickness of silica gel 7G
Solvent: 3% Methanol in chloroform.

C. Ascending column chromatography on a Sephadex LH20 gel column. The column was equilibrated and eluted with chloroform except fraction V which was eluted with 50% methanol in chloroform.
I (2) green yellow, orange. II, green (Chl a + Chl b). III, orange. IV, deep yellow. V, (2) pale green, green.

Fig. 13. The spectra of fractions I and II separated by Sephadex LH20 gel column chromatography of pigments extracted from air control and 50 ppm ethylene-treated spinach leaves. Pigment fractions were in chloroform solution during scanning of the spectra.

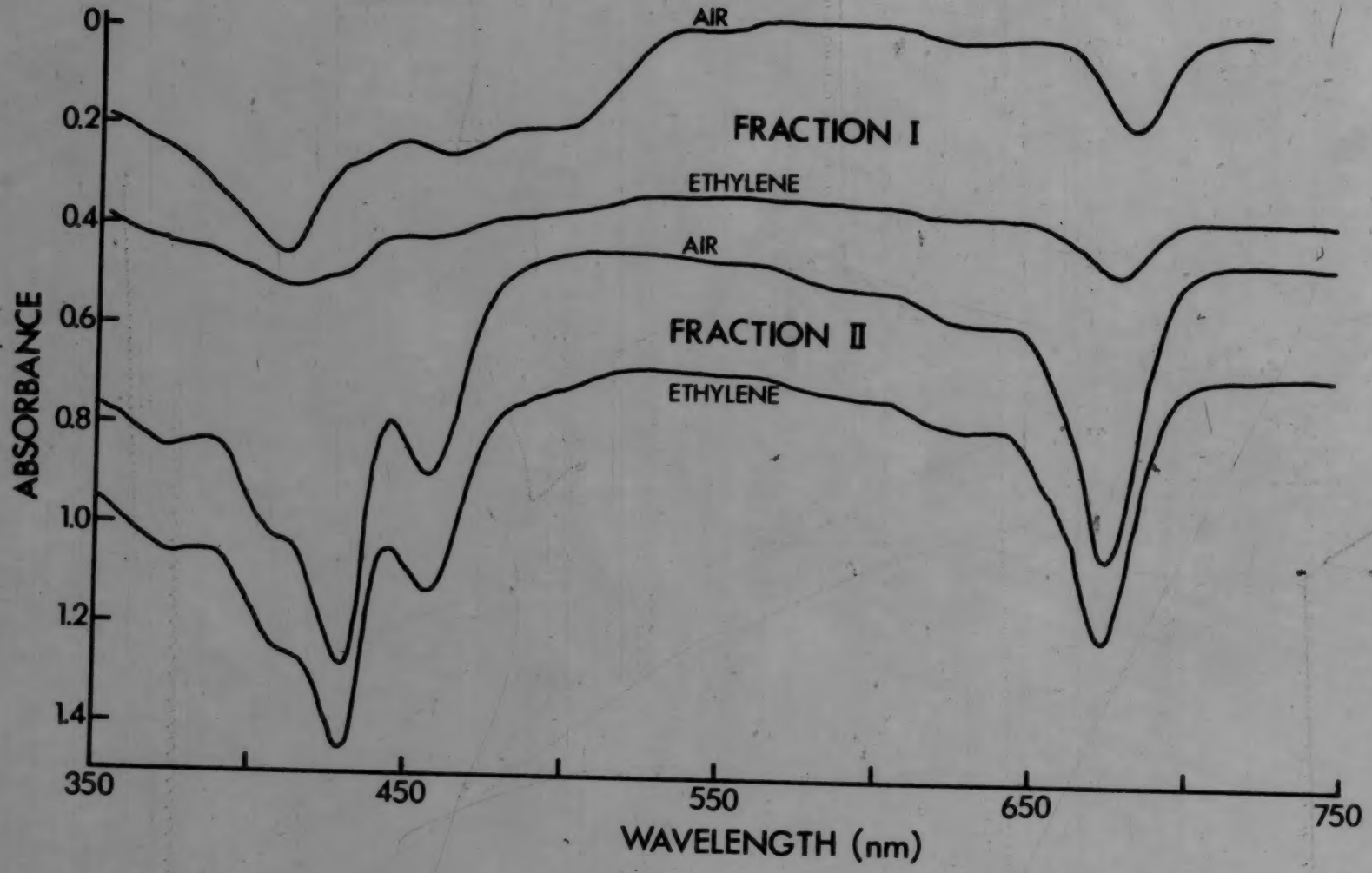
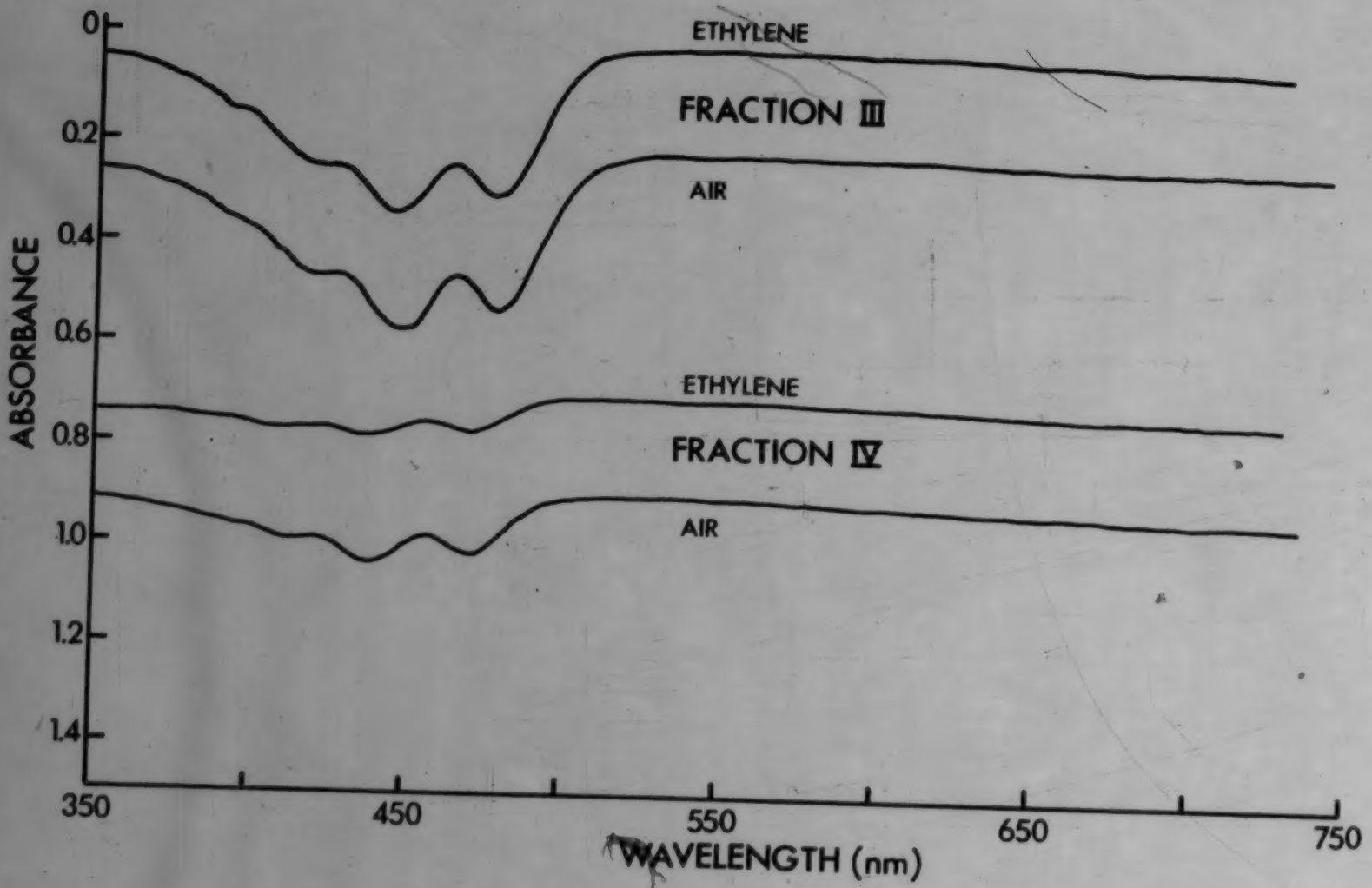

AIR
FRACTION I
ETHYLENE
AIR
FRACTION II
ETHYLENE
ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
350
450
550
650
750
WAVELENGTH (nm)

Fig. 14. The spectra of fractions III and IV separated by Sephadex LH20 gel column chromatography for pigments extracted from air control and 50 ppm ethylene treated spinach leaves. The spectra of the pigments fractions were recorded in chloroform solution.


ETHYLENE
FRACTION III
AIR
ETHYLENE
FRACTION IV
AIR
ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
350
450
550
650
750
WAVELENGTH (nm)

two bands, which did not separate well, were eluted together to form fraction I. The visible spectrum of the fraction measured was recorded in Fig. 13. Spectra of the other fractions of air- and ethylene-treated samples were not different as shown in Fig. 14. The pigment contents of leaves treated with air or 50 ppm ethylene in air are shown in Table 7. The respective absorption maximum was selected for measurement of absorbance for each fraction. Total chlorophyll content of ethylene-treated sample was about 72% of that of the air control. Carotenes and other carotenoids of ethylene-treated leaves were also decreased compared to that of air-treated leaves.

B. Studies on the Protection of Chlorophyll against Ethylene Action

1. Carbon dioxide

Figure 15 clearly reveals that the effect of CO_2 is not directly proportional to its concentration. With 50 ppm ethylene and without CO_2, the chlorophyll content decreased in three days to 60% of the control kept in CO_2-free air. Then, an addition of CO_2 at a concentration as low as 0.009%, counteracted very efficiently the ethylene-induced chlorophyll degradation. As we have not applied lower concentrations, we are not able to extrapolate the threshold value at which CO_2 would no longer be effective. Then, surprisingly, as the CO_2 concentration increased, its "protective" effect decreased, and at 10% it acted rather synergistically with ethylene; at this CO_2 concentration the chlorophyll content was minimal. With gradually higher concentrations, the chlorophyll degradation decreased again. It should be noted, however, that in high concentrations of CO_2, the leaves

TABLE 7. The effect of 50 ppm ethylene on pigments of spinach leaves

Fractions	Air control (O.D. reading)	Ethylene (O.D. reading)
Fraction I	A_{676*} = 0.494	A_{676} = 0.375
	A_{680*} = 0.706	A_{680} = 0.546
Fraction II (50 ml) chlorophylls	A_{649**} = 0.562	A_{649} = 0.386
	A_{665**} = 1.002	A_{665} = 0.698
Fraction III (25 ml) carotenes	A_{446*} = 0.660	A_{446} = 0.531
Fraction IV (25 ml) carotenoids	A_{437*} = 0.132	A_{446} = 0.092
Fraction V (10 ml) chlorophyllides, pheophorbides	A_{660}= 0.015 (trace)	A_{660} = 0.012 (trace)

* Maxium absorption of fractions. ** The wavelengths used for chlorophyll determination according to the formula of Vernon (1960).

Pigments from air- and ethylene-treated spinach leaves in a continuous gas flow for three days were separated by Sephadex LH20 gel column chromatography. The light intensity during treatment was 50 ft candles.

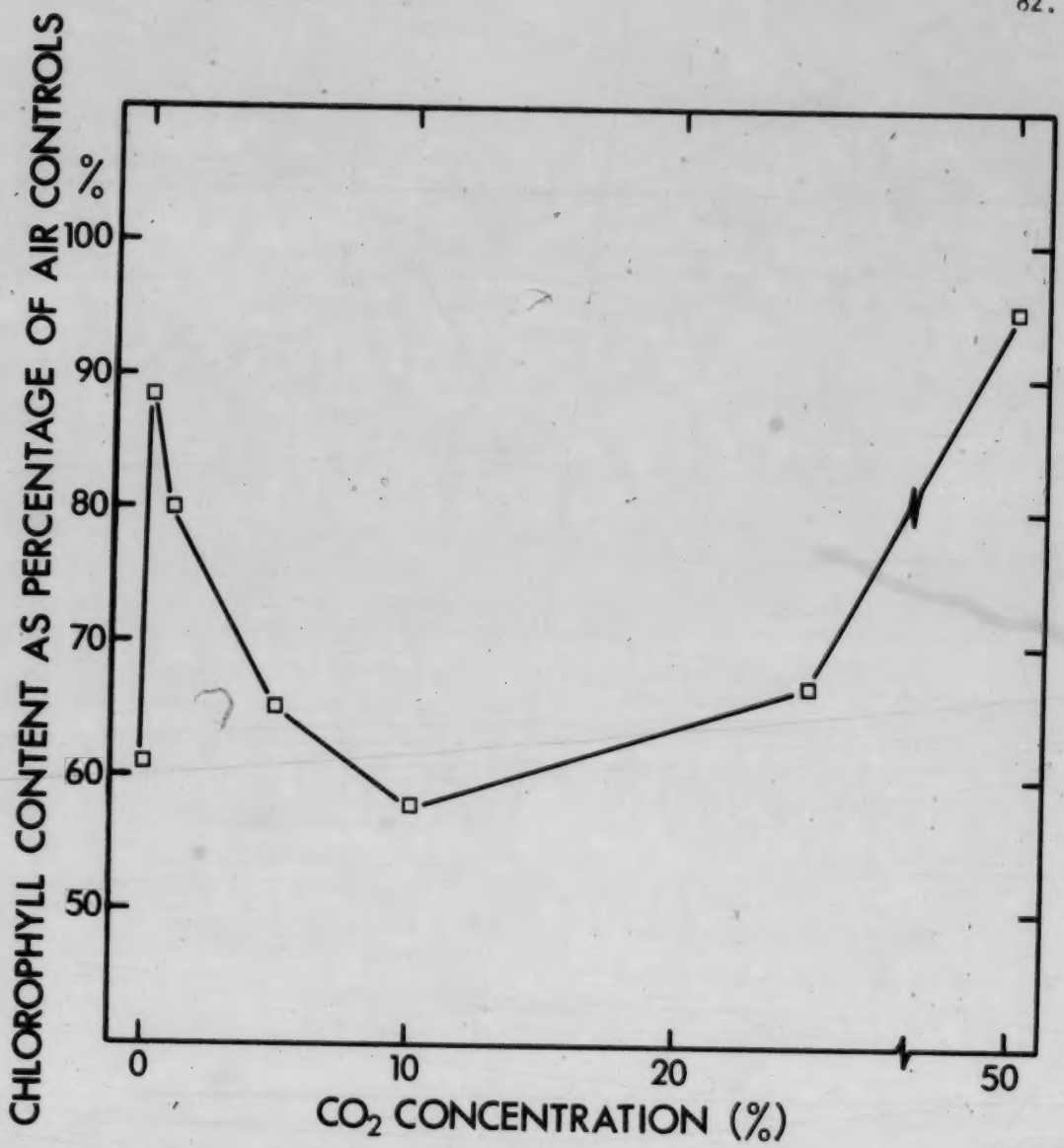


Fig. 15. The effect of CO_2 on protection of chlorophyll of spinach leaves against 50 ppm ethylene treatment.

50 ppm ethylene was applied throughout the experiment with a continuous gas flow. The time of treatment was three days.

CO_2 concentrations: 0.009%, 1%, 5%, 10%, 25%, and 50%.

underwent some wilting, which is an indication of some detrimental effect of the gas on the physiology of the leaves.

2. Cytokinins

Table 8 shows that the effect of 50 ppm ethylene on senescence, in terms of chlorophyll destruction, was reduced by the addition of antisenescent agents such as kinetin and N-6-benzyl adenine (NBA). NBA gave only a weak protective effect. Although higher chlorophyll contents in spinach leaves were found with the application of both kinetin and NBA, than that with ethylene alone, the protective effect decreased with the highest concentration of cytokinins applied, *i.e.* 100 µg/ml. On the contrary, kinetin at low concentrations proved to be very effective. There was a significant difference between the results with 50 ppm ethylene and 50 ppm ethylene with 10 µg kinetin/ml.

In order to check whether or not the translocation of cytokinins enhanced the effectiveness of chlorophyll protection, disks of spinach leaves were placed on the surface of cytokinins solutions. Assays were done again with or without 50 ppm ethylene. The effectiveness of protection of chlorophyll by cytokinins was only improved slightly as shown in Table 9. A comparison of the effects of chlorophyll content of spinach leaves and their disks treated with cytokinins are presented in Table 10. It can be seen that the protective effect of cytokinin on chlorophyll with leaf disks was somewhat higher than with whole leaves.

Ethylene production was not reduced by the addition of either kinetin or NBA in the solution in which the detached whole spinach leaves (about ten grams) were dipped. The production of ethylene in the presence of kinetin (2 µg/ml) and NBA (2 µg/ml) was higher almost at all times during the experiments (except at the first twenty minutes

TABLE 8. Effects of cytokinins on the chlorophyll content of detached spinach leaves treated with 50 ppm ethylene in air for three days

	Chlorophyll Content			
	In presence of kinetin		In presence of NBA	
	% air	% C_2H_4	% air	% C_2H_4
Air control	100		100	
+ Cytokinins, 2 µg/ml	101		103	
+ Cytokinins, 10 µg/ml	101		109	
50 ppm Ethylene	70	100	78	100
+ Cytokinins, 1 µg/ml	79	113	85	104
+ Cytokinins, 2 µg/ml	87	124	82	102
+ Cytokinins, 10 µg/ml	105	150	84	103
+ Cytokinins, 100 µg/ml	96	137	81	102

Spinach leaves were from four weeks old plants grown in growth chambers. Chlorophylls were extracted and estimated as described earlier under Materials and Methods (B. 2). Treatment with ethylene was a continuous gas flow. Air control was arbitrarily called "100%".

TABLE 9. Effects of cytokinin on the chlorophyll content of leaf disks from ethylene-treated spinach leaves

	Chlorophyll content			
	In presence of kinetin		In presence of NBA	
	% air	% C_2H_4	% air	% C_2H_4
Air control	100		100	
Cytokinin (2 µg/ml)	103		104	
50 ppm Ethylene	71	100	74	100
Cytokinin (0.2 µg/ml)	72	100	75	100
Cytokinin (2 µg/ml)	93	130	82	110
Cytokinin (20 µg/ml)	96	135	82	110

Spinach leaves were from four weeks old plants grown in the growth chamber. The leaves were treated with ethylene in a continuous gas flow for three days. Air control was arbitrarily called "100%".

TABLE 10. Comparison of the effects of cytokinins on the chlorophyll of whole leaves and their disks*

	Whole leaves			Leaf disks		
	Chl content	Cytokinin effect		Chl content	Cytokinin effect	
		% air	% Ethylene		% air	% Ethylene
Air control	1.75	100		2.66	100	
+ Kinetin, 2 µg/ml	1.76	105		2.67	118	
+ NBA, 2 µg/ml	1.80	115		3.28	145	
50 ppm Ethylene	1.35	77	100	2.07	91	100
+ Kinetin, 2 µg/ml	1.52	87	112	2.40	106	116
+ NBA, 2 µg/ml	1.44	82	107	2.58	110	120

Whole spinach leaves, or their disks were treated with air or 50 ppm ethylene under continuous flow for three days at a light intensity of 50 ft candles. Chlorophyll content is expressed as mg/g fresh wt. Air control was arbitrarily called "100%".

described as zero hour in Table II) than that without the presence of cytokinins (controls). On Day 3, ethylene production by kinetin and NBA treated samples was three and six times respectively that of the control. The production of ethylene by the disks of spinach leaves was higher after two, five, eighteen and twenty-four hours of kinetin treatment whereas in NBA treated samples, ethylene production after only two and three hours was higher than that of the control. Finally, it should be pointed out that the ethylene production by the disks was always much higher than that of whole leaves.

C. Effects of Ethylene on the Photosynthetic Apparatus and Activity

1. Isolation of chloroplast from spinach leaves

In the present study, chloroplasts isolated from spinach leaves purchased from the local city market or grown in the University greenhouse or growth chamber during the winter months did not exhibit the normal biochemical activity in terms of O_2 evolution or $^{14}CO_2$ fixation upon the addition of Na_2CO_3 or $Na_2C^{14}O_3$ respectively. However, the Hill reaction was operative when ferricyanide was supplied as an exogenous electron acceptor. The effect of ethylene on the Hill reaction in isolated chloroplasts will be reported later. The results of CO_2 fixation to be presented here were obtained using chloroplasts isolated from the spinach leaves of plants grown on the University farm or purchased from the city market during summer.

(i) Morphology of isolated chloroplasts

The differences in morphology of the chloroplasts arise from the use of a meat grinder, mortar and pestle and semi-micro vessel Waring blendor. The preparation of spinach leaves and the conditions of

TABLE II. The effects of kinetin and NBA on ethylene production by detached whole spinach leaves and their disks

Collection time	Whole leaves (nl C_2H_4/g fresh wt/h)			Disks (nl C_2H_4/g fresh wt/h)		
	In presence of water	In presence of kinetin	In presence of NBA	In presence of water	In presence of kinetin	In presence of NBA
0 hour	9.0	2.3	2.8	52.9	38.9	38.5
2 hours	3.7	4.4	4.9	36.8	50.0	46.5
5 hours	1.6	2.2	2.9	21.4	27.6	29.1
18 hours	1.0	1.1	1.9	4.2	6.6	3.0
Day 1	0.8	1.0	1.4	2.3	7.6	2.4
Day 2	0.9	1.1	1.0	7.3	6.9	2.0
Day 3	0.4	1.3	2.5	6.0	4.2	0.2

Spinach leaves were from 4 weeks old plants grown in a growth chamber. The concentration of cytokinins (kinetin and NBA) was 2 µg/ml. A system with a light intensity of 50 ft candles and a continuous gas flow at room temperature was carried out during the treatment. Ethylene was collected for 20 minutes and measured by gas liquid chromatography as described in Materials and Methods (B. 1, I&ii). The results are the average of duplicate experiments.

isolation were similar in these three different homogenization techniques. The percentage breakage of chloroplasts as apparent under the light microscope was for the meat grinder, 30%, mortar and pestle, 15%, and Waring blendor, 10%. However, the rate of O_2 evolution was similar for all the three methods as will be shown later.

Since the chloroplast membranes cannot be seen under the light microscope, electron microscopy was used to determine the integrity of the membranes. Pictures of chloroplasts isolated from spinach grown in the growth chamber are shown in Fig. 16. Although the thylakoid and outer envelope membranes of isolated chloroplasts were intact, the organization of the chloroplasts seemed to be altered slightly after isolation by comparing the results obtained from spinach leaf specimens (Fig. 17). The starch granules as seen in Fig. 16a were different from those in Fig. 16b. They could be forms of different stages. It is interesting to note the difference in staining of starch granules.

Chloroplasts isolated from spinach grown in the growth chamber were further purified by zonal sucrose gradient centrifugation. The chloroplasts fraction corresponding to 40.5% of sucrose concentration was collected. The density of chloroplasts was 1.1765 (D^{20}).

(ii) Studies on the Hill reaction

a. Oxygen evolution by isolated chloroplasts

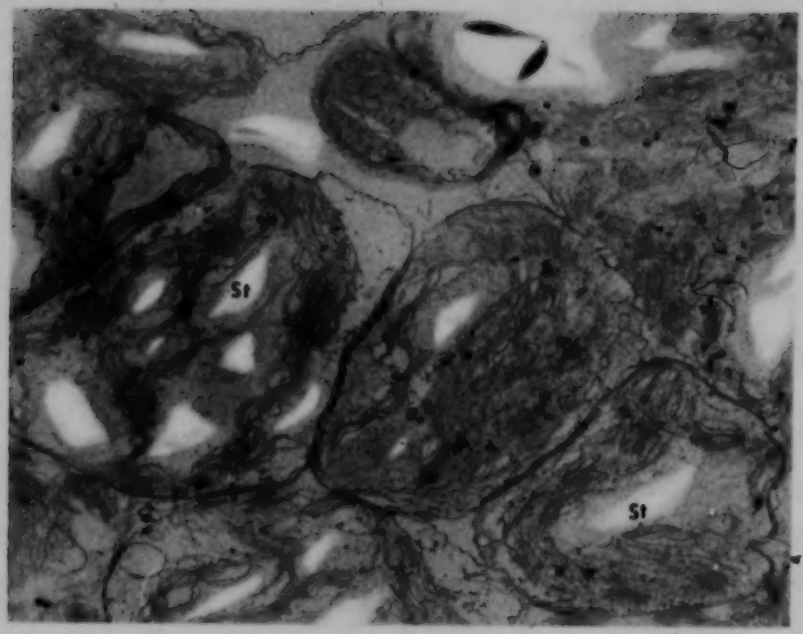
The O_2 evolution of chloroplasts isolated by three different ways appeared to be similar when Na_2CO_3 was used as a carbon dioxide source, as shown in Table 12.

The stability of the process of O_2 evolution by the chloroplasts isolated by the three methods was also studied. None of these evolved O_2 after five hours of storage in HEPES buffer at 0°C. The addition of

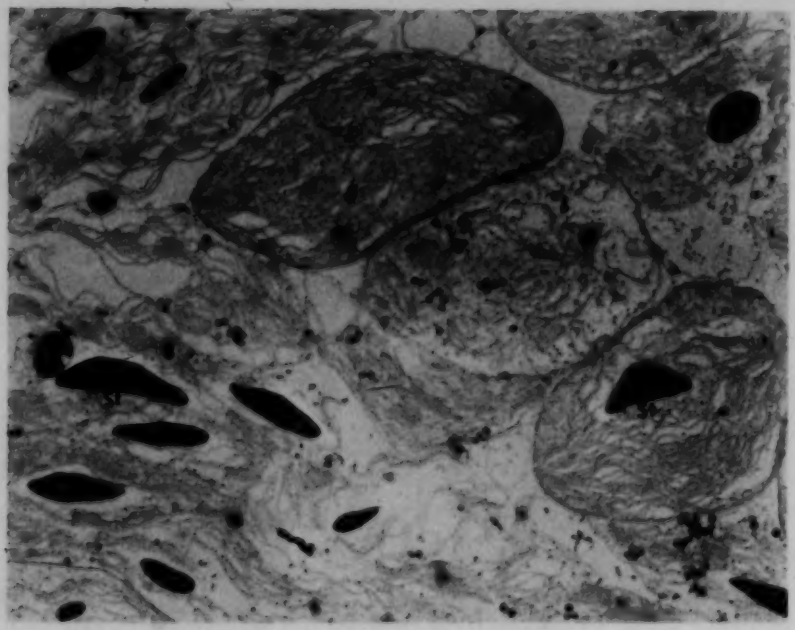
Fig. 16. Electron micrograph of isolated chloroplasts from fresh spinach leaves grown in growth chamber.

Isolated chloroplasts were fixed in glutaraldehyde and osmium tetraoxide. These were subsequently stained with uranyl acetate and lead citrate and examined in the electron microscope, Philip 200. The ultrastructure of isolated chloroplasts considered to be normal except for a slight alteration of organization of thylakoid membranes, probably because of the preparation of isolated chloroplasts for electron microscopy study. Figures 16a and 16b probably represent chloroplasts of different stages because of showing different response of electron reflection by the starches.

St - starch granule



a



b

Fig. 17. Electron micrograph of chloroplasts in spinach leaf.

Spinach was grown in growth chamber. A 5 cm leaf was prepared for electron microscope examination according to the method mentioned in Materials and Methods (C.2.iii.a, p. 52)

M - mitochondria

CW - cell wall

Th - thylakoids

Os - osmiophilic globules

St - starch

M
CW
S
CW
M
S

TABLE 12. Oxygen evolution by isolated chloroplasts

Mode of leaf maceration	Oxygen evolution (μmoles O_2/mg Chl/min)
Meat Grinder	0.883
Semimicro Waring Blendor Vessel	0.888
Mortar and Pestle	0.919

Spinach was bought from a local market during the summer. The chloroplasts were isolated in MES buffer and oxygen evolution was studied in TRICINE buffer containing Na_2CO_3 as a source of CO_2 in the oxygen monitor at 20°C and at a light intensity of 2500 ft candles.

Master buffer (A): $NaNo_3$ (2 mM), $MnCl_2$ (1 mM), $MgCl_2$ (1 mM), K_2PO_4 (0.5 mM), EDTA (2 mM), Sorbitol (0.33 M).

Isolation (MES) buffer: A + MES (0.05 M) + 0.02 M NaCl, pH 6.1.

Reaction (TRICINE) buffer: A + TRICINE (50 mm) + $Na_4P_2O_4$ (5 mM), pH 8.1.

0.5 mM of NADP could restore the activity of O_2 evolution to some extent (Fig. 18). In the figure, the reaction mixture without NADP showed a lag period of three to four minutes depending on the length of storage of the chloroplasts. But the reaction mixture with NADP did not show any lag period. The O_2 evolution increased immediately after illumination. However, the rate of O_2 evolution was reduced after eight minutes with or without addition of $NADP^+$.

The results of the study on the O_2 evolution by spinach chloroplasts as affected by the length of storage, up to five days, of spinach plants at 4°C are shown in Table 13. As expected, the activity decreased with increasing storage time. When the leaves were transferred from 4°C to 0°C, without freezing, oxygen evolution increased over that at 4°C.

The photosynthetic O_2 evolution by "chloroplasts" isolated from spinach grown in the greenhouse, growth chamber, or purchased from the local city market during the winter months showed no O_2 evolution at all when carbon dioxide (actually, Na_2CO_3) was added, while the Hill reaction in isolated chloroplasts proceeded very well when ferricyanide was added. The quotation of "chloroplast" is specially assigned to in indicate that the chloroplast may not be intact.

b. Effects of ethylene on the Hill reaction

The "chloroplasts" isolated from ethylene-treated spinach leaves also carried out the Hill reaction only when ferricyanide was used as an electron acceptor. There was no effect of ethylene (50 ppm) on O_2 evolution by isolated "chloroplasts" from spinach leaves that had either no or one day treatment with ethylene (Table 14 and Fig. 19). However,

Fig. 18. Oxygen evolution by isolated chloroplasts

Legend: o Chloroplasts immediately after isolation.

Δ Chloroplasts in HEPES buffer at 0°C for 5 hours with addition of 0.5 mM $NADP^+$ just before the light was turned on.

o Chloroplasts in HEPES buffer at 0°C for 4 hours.

x Chloroplasts in HEPES buffer at 0°C for 5 hours.

Master buffer (A): $NaNO_3$ (2 mM), $MnCl_2$ (1 mM), $MgCl_2$ (1 mM), K_2PO_4 (0.5 mM), EDTA (2 mM), Sorbitol (0.33 M).

Isolation (MES) buffer:(B): A + MES (0.05 M) + 0.02 M NaCl, pH 6.1.

Suspension (HEPES) buffer (C): A + HEPES (50 mM) + 0.02 M NaCl, pH 6.7.

Reaction (TRICINE) buffer (D): A + TRICINE (50 mM) + $Na_4P_2O_7$ (5 mM), pH 8.1.

The study of oxygen evolution by isolated chloroplasts was done in oxygen monitor at 20° ± 1°C with a light intensity of 2500 ft candles and Na_2CO_3 was added as a CO_2 source.

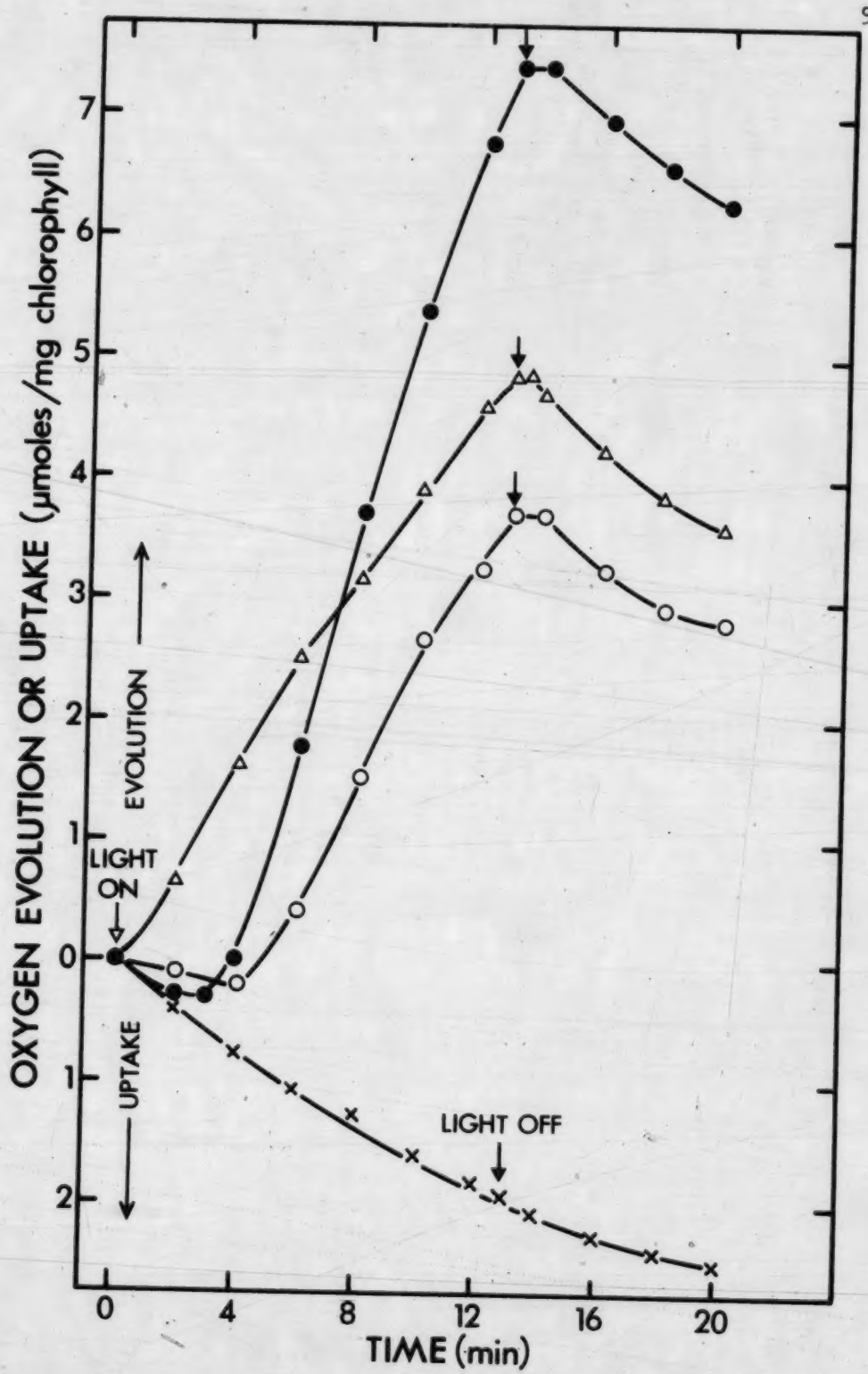

OXYGEN EVOLUTION OR UPTAKE (μmoles/mg chlorophyll)
7
6
5
4
3
2
1
0
1
2
EVOLUTION
LIGHT ON
UPTAKE
LIGHT OFF
0
4
8
12
16
20
TIME (min)

TABLE 13. The photosynthetic O_2 evolution by chloroplasts isolated from spinach leaves stored in plastic bags at 4°C

Length of leaf storage	Oxygen evolution (µmoles O_2/mg Chl/min)
Chloroplasts isolated on the day of harvest	1.325
3 days	0.490
3 days at 4°C, 1 day at 0°C without freezing	0.736
5 days	0.135

The spinach leaves were obtained from the University Farm. The isolation of chloroplasts and the measurement of oxygen evolution were as described earlier under Materials and Methods (C. 2. ii, p.48). The chloroplasts were isolated in MES buffer and oxygen evolution was studied in TRICINE buffer containing Na_2CO_3 as a source of CO_2 in oxygen monitor at 20°C and at a light intensity of 2500 ft candles.

TABLE 14. Effects of ethylene on Hill reaction of "chloroplasts" isolated from spinach leaves without previous ethylene treatment

Experiments	Oxygen evolution (μmoles O_2/mg Chlorophyll/h)	
	In presence of air	In presence of ethylene
1	113.8	93.2
2	106.0	118.0
3	122.5	109.2
4	159.5	160.2
Average	120.2	125.5
Standard deviation	±12.2	±14.7

Spinach plants were grown in a growth chamber. "Chloroplasts" were isolated in MES buffer and oxygen evolution was studied in TRICINE buffer containing Na_2CO_3 as a source of CO_2 in the oxygen monitor at 20°C and at a light intensity of 2500 ft candles. There was no difference in Hill reaction of isolated "chloroplasts" in the presence of air and ethylene. "Chloroplasts" were isolated from spinach leaves without previous ethylene treatment.

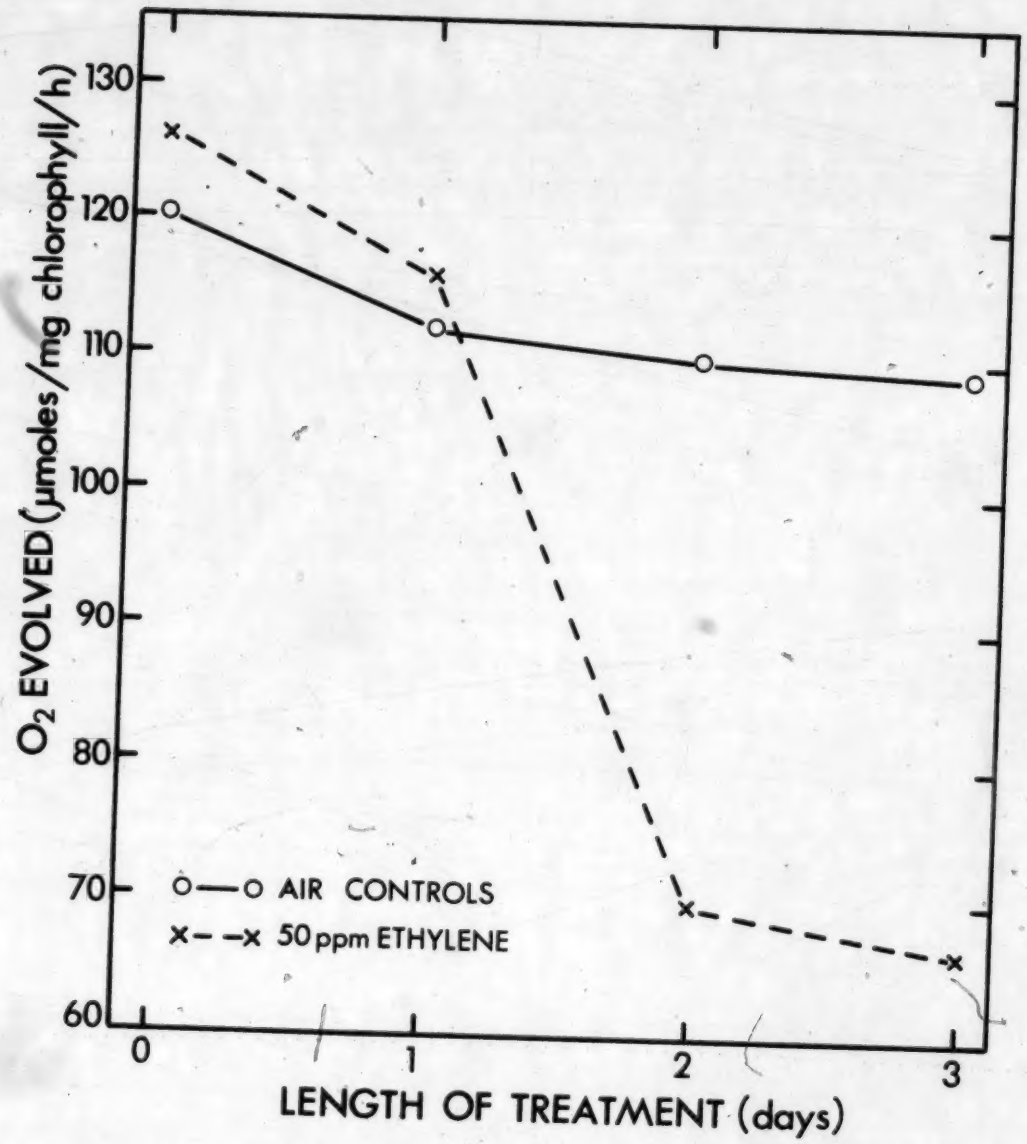


Fig. 19. Effects of ethylene on Hill reaction of "chloroplasts" isolated from detached spinach leaves previously treated with 50 ppm ethylene.

the rate of the Hill reaction of isolated chloroplasts from leaves treated two or three days with 50 ppm ethylene was 60% of that from air-treated leaves (Fig. 19).

The study of the Hill reaction of isolated "chloroplasts" from ethylene- and air-treated spinach leaves was repeated in the presence of air and ethylene respectively, in order to find out if the effect of ethylene could be reversed. The effects of switching atmosphere during the progress of Hill reaction on the oxygen evolution was negligible. This would suggest that the effects of ethylene on the chloroplasts are probably irreversible.

(iii) Studies on the CO_2 fixation

a. Photosynthetic CO_2 fixation by isolated chloroplasts

The $^{14}CO_2$ fixation by chloroplasts isolated from spinach grown at the University farm is shown in Fig. 20. In this study, an initial lag or induction period (usually three to six minutes) was constantly observed before reaching the maximum rate of $^{14}CO_2$ fixation. As expected, there was $^{14}CO_2$ fixation even after the light was turned off.

With isolated chloroplasts from spinach leaves grown in the University farm during summer, the photosynthetic $^{14}CO_2$ fixation resulted in about 50% ^{14}C incorporation of total $Na_2^{14}CO_3$ added. The results are recorded in Table 15. The chloroplasts from spinach leaves washed and stored at 0°C without freezing for one day had greatly enhanced $^{14}CO_2$ fixation compared with the chloroplasts isolated from fresh spinach. During summer, the spinach from the local city market had high $^{14}CO_2$ fixation activity as well. A maximum of 23% ^{14}C incorporation was obtained between six to twelve minutes of exposure to light

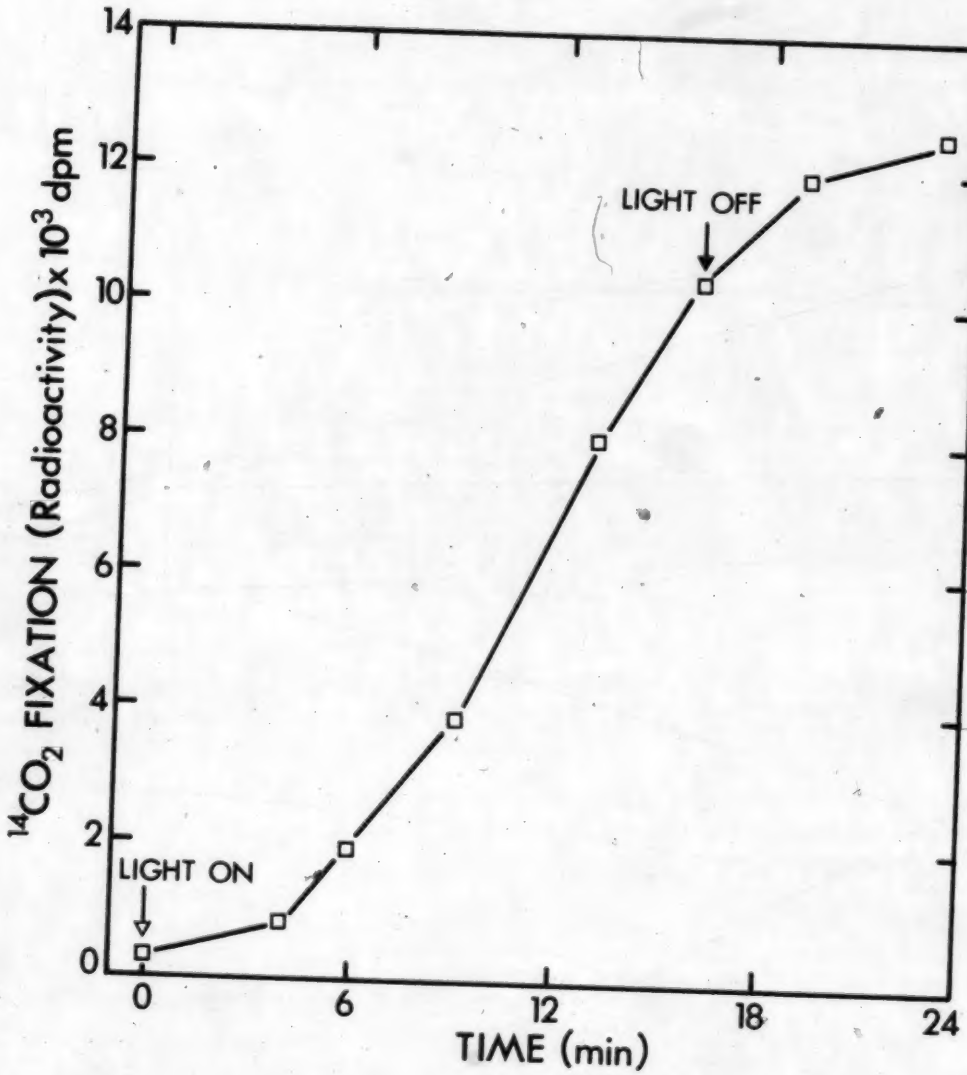


Fig. 20. $C^{14}O_2$ fixation of chloroplasts isolated from spinach grown in the University Farm.

TABLE 15. Photosynthetic CO_2 fixation by chloroplasts from spinach grown in the University Farm

	CO_2 fixation (% ^{14}C incorporation)	
	By chloroplasts isolated from fresh spinach leaves (no previous chilling)	By chloroplasts isolated from 1 day old spinach leaves stored at 0°C
Light on (min)		
3.5	--	7.3
4.0	4.6	--
6.0	8.2	11.9
9.0	13.7	--
10.0	--	26.2
15.0	17.2	45.2
Light Off (min)		
3.0	17.4	--
4.0	--	49.2
6.0	17.8	49.9

The chloroplasts were incubated with $Na_2{}^{14}CO_3$ in a reaction vessel of the oxygen monitor. The method is described under Materials and Methods. CO_2 fixation was calculated as % of ^{14}C incorporation into chloroplasts compared to the total $Na_2{}^{14}CO_3$ added. The data are the average of duplicate experiments with the results at a minimal variation.

(Table 15). By comparison, the chloroplasts isolated from spinach leaves stored at 4°C for varying lengths of time from one day to one week showed a decreasing CO_2 fixation activity (Table 16). Chloroplasts isolated from fresh leaves (leaves that were chilled to 4°C for two hours before isolation) had three times more activity than those from leaves stored at 4°C for one day.

2. Effect of ethylene on CO_2 fixation by detached spinach leaves

The net CO_2 assimilation under high light intensity (2500 ft candles) by spinach leaves previously treated either with ethylene or air (in presence of 50 ft candles light) was reduced by treatment with 50 ppm ethylene (Fig. 21). On the basis of chlorophyll contents, the amounts of CO_2 fixed at Day 0, 1, 2, and 3 were 88, 79, 50, and 35% of the air control respectively. In spinach leaves previously treated with air, the trend of CO_2 assimilation was similar to the performance of Hill reaction of isolated chloroplast (Fig. 19); a decrease in activity during the first day, followed by a constant level for the rest of the treatment. However, in ethylene-treated samples, the CO_2 fixation activity decreased with time (Fig. 21).

When results of CO_2 assimilation were based upon leaf area, the rates of net CO_2 taken up by ethylene-treated (50 ppm) samples were 83, 67, 32, and 14% of air controls after zero, one, two, and three days of exposure respectively. When based on the chlorophyll content, the CO_2 assimilation was higher than when based on leaf area (Fig. 22). But the trend remained the same with higher CO_2 fixation in air-treated samples. It appears from the above results that the effect of ethylene on CO_2 fixation was more extensive than the mere destruction of chlorophylls.

In the presence of 50 ppm ethylene, net CO_2 fixation by spinach

TABLE 16. Photosynthetic CO_2 fixation* by chloroplasts isolated from spinach leaves stored for various lengths of time

Length of illumination (min)	Chloroplasts isolated from fresh spinach leaves	Storage time			
		1 day	2 days	5 days	1 week
3	1.0	0.9	0.2	--	0.1
4	--	--	--	0.2	--
6	6.8	2.0	0.5	0.4	0.2
9	14.1	3.0	0.8	--	0.5
10	--	--	--	0.9	--
12	22.5	6.5	1.4	--	0.5
13	--	--	--	1.9	--
15	23.9	12.9	--	--	0.8

*CO_2 fixation was measured as % incorporation of $^{14}CO_2$ compared to total radioactivity of $Na_2{}^{14}CO_3$ added.

The chloroplasts were incubated with TRICINE buffer containing 15 µmoles Na_2CO_3 with approximately 10 µc of ^{14}C in a reaction vessel of the oxygen monitor at 20°C and a light intensity of 2500 ft candles. Spinach purchased from the local city market during the summer was used and stored in a plastic bag at 4°C.

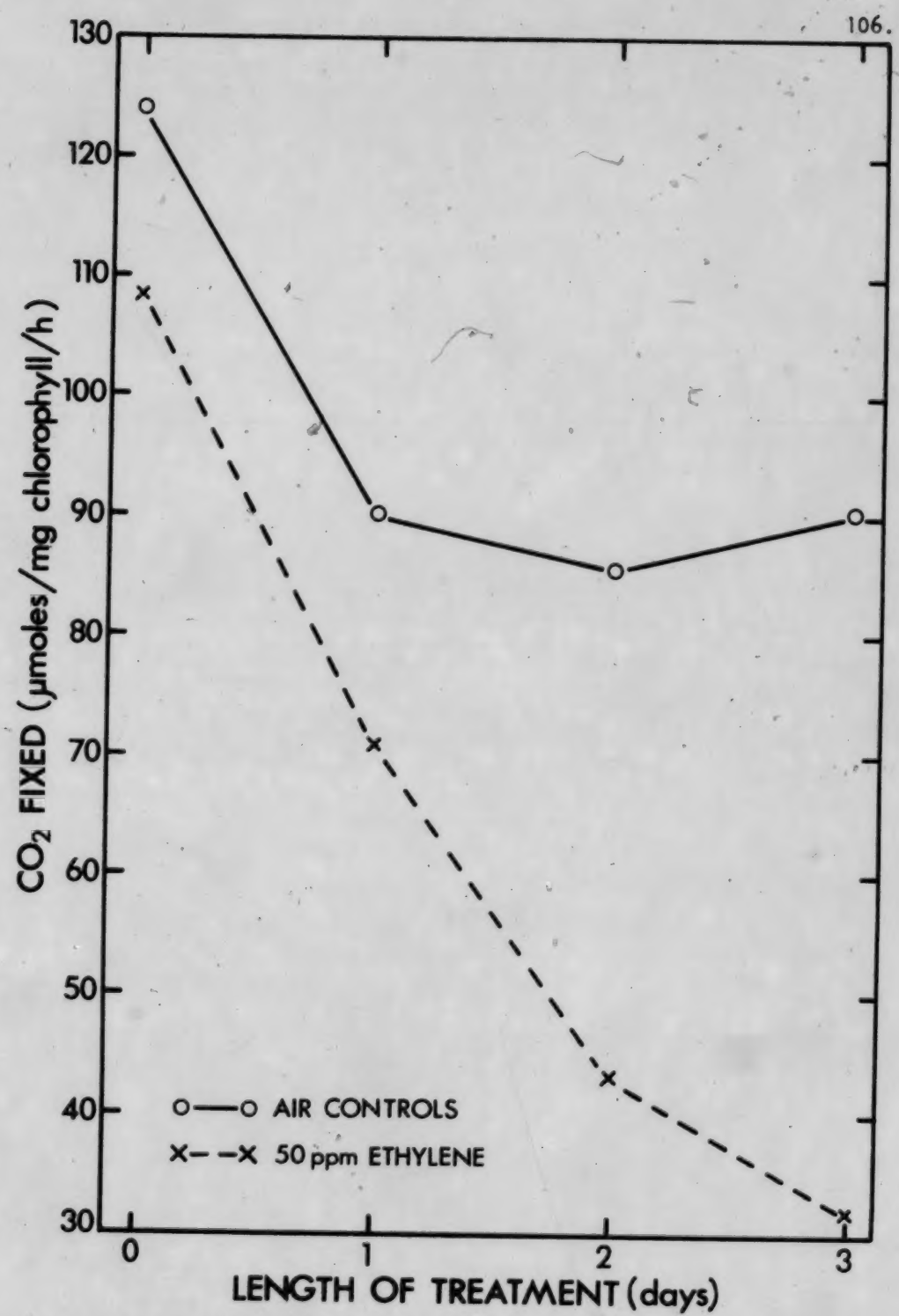


Fig. 21. Net CO_2 assimilation of air- and 50 ppm ethylene-treated spinach leaves at high light intensity (2500 ft candles). Both kinds of leaves were previously treated at low light intensity of 50 ft candles.

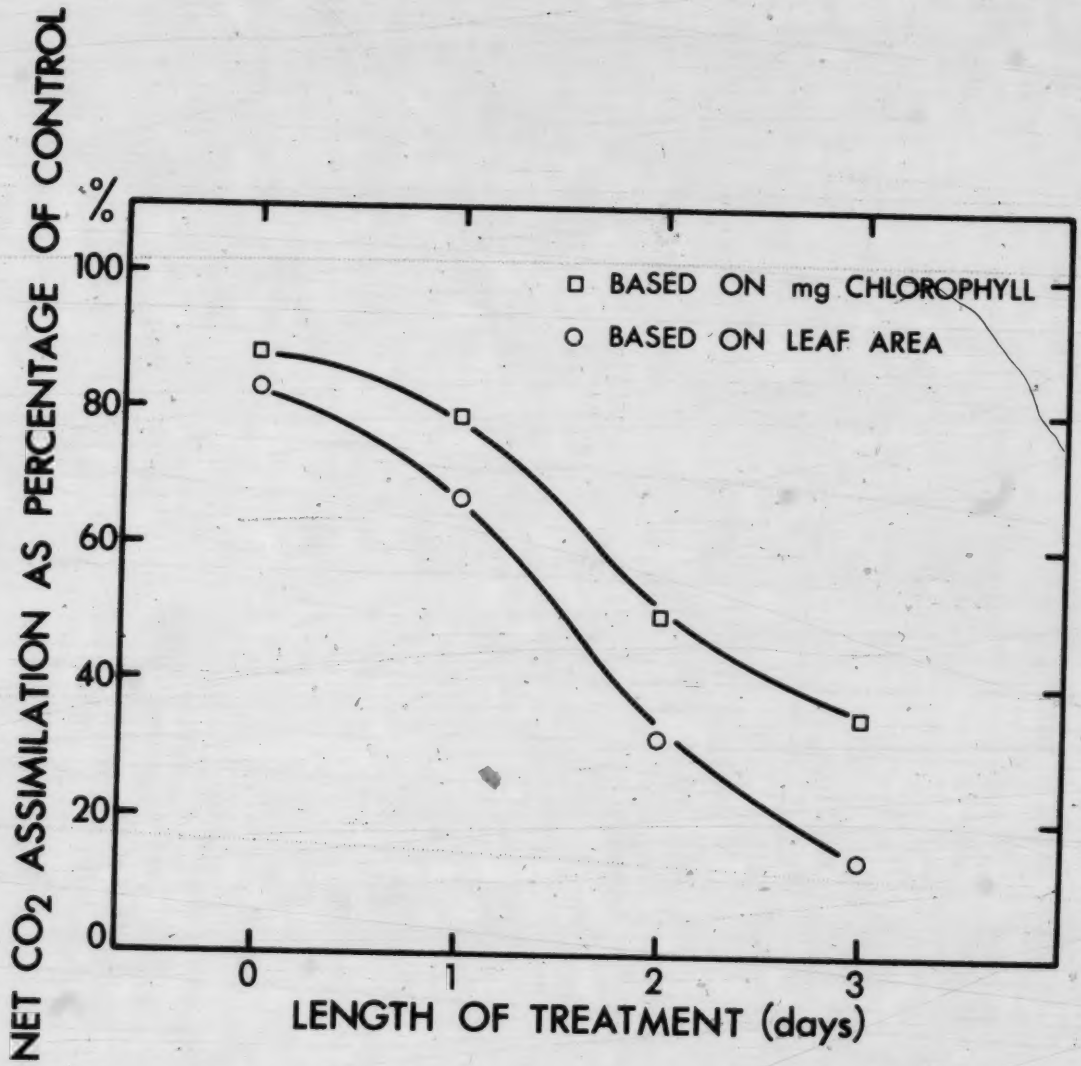


Fig. 22. The effect of 50 ppm ethylene on net CO_2 assimilation of detached spinach leaves at a light intensity of 2500 ft candles.

leaves under low light intensity (50 ft candles) was higher than for air-treated leaves (Fig. 23). The spinach leaves were previously treated with ethylene or air under low light intnesity (50 ft candles). The CO_2 fixation by ethylene-treated samples decreased slightly initially and then increased after one day of treatment, whereas in air-treated leaves, it decreased until Day 2 before increasing again. The difference between ethylene- and air-treated samples were 2.7, 5.4, 17.6, and 15.5 μmoles CO_2/mg chlorophyll/h for Day 0, 1, 2, and 3 respectively. The effect of ethylene under low light intensity was thus completely different from the effect under high light intensity (2500 ft candles). For comparison of double reciprocal plot of light intensity and net CO_2 assimilation in the presence or absence of ethylene (see Fig. 24) might be explained by a lowering of the light saturation point of spinach leaves by ethylene.

The effect of ethylene (50 ppm) on the net CO_2 fixation by spinach leaves prviously treated with 50 ppm ethylene in the dark was also studied under high light intensity (2500 ft candles) (Fig. 25). Although the net CO_2 fixation by air-treated samples was higher than that of ethylene-treated samples, the differences between these values were not as great as those of spinach leaves treated at 50 ft candles.

3. Effects of ethylene on dark respiration of spinach leaves

Ethylene is generally believed to increase respiration of plant tissues. Spinach leaves are not exception. Therefore, as spinach leaves were used in this study, it was necessary to look at the effect of ethylene on the rate of respiration.(see Table 17). The values of the dark respiration of ethylene-treated samples, measured as CO_2 evolution, were always higher than those of the air controls for all lengths of treatments for about two to three times.

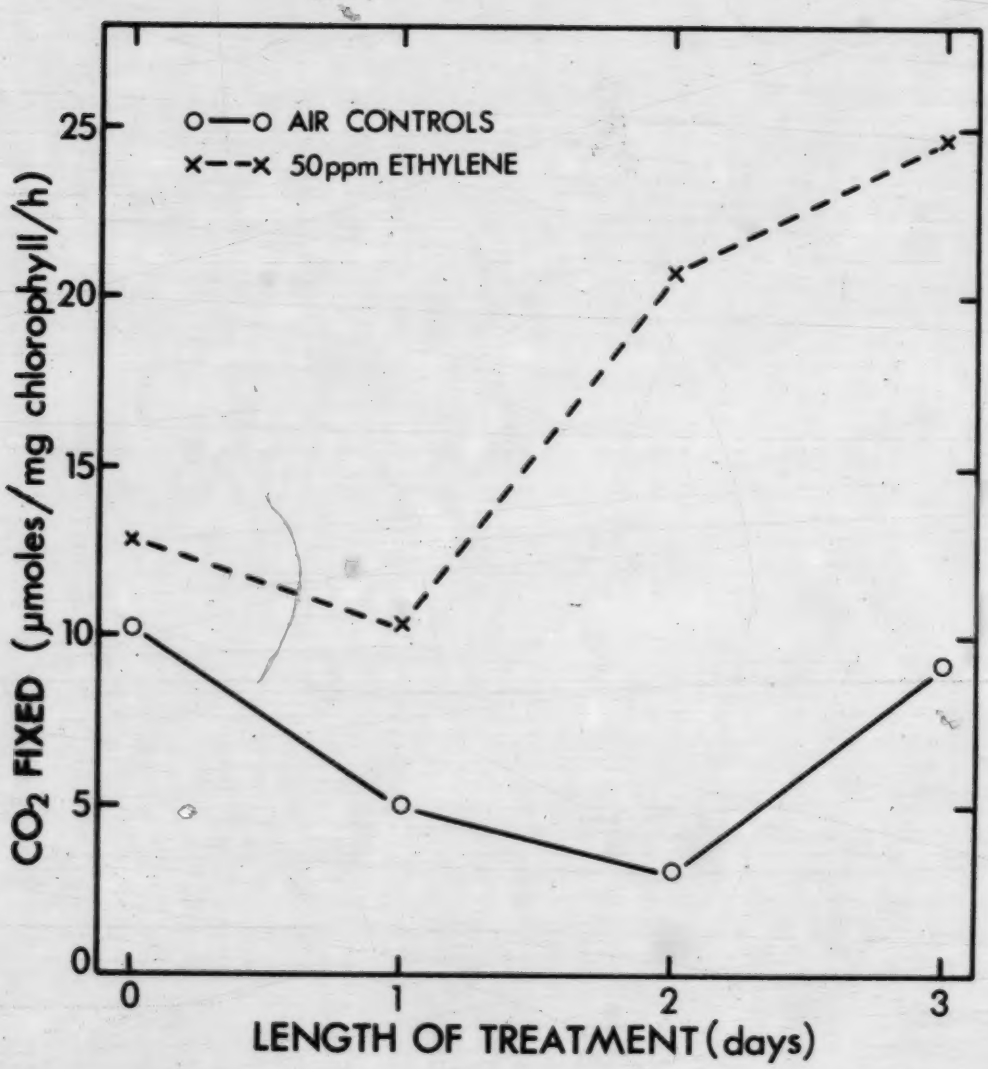


Fig. 23. Net CO_2 assimilation of air- and 50 ppm ethylene-treated spinach leaves at low light intensity (50 ft candles).

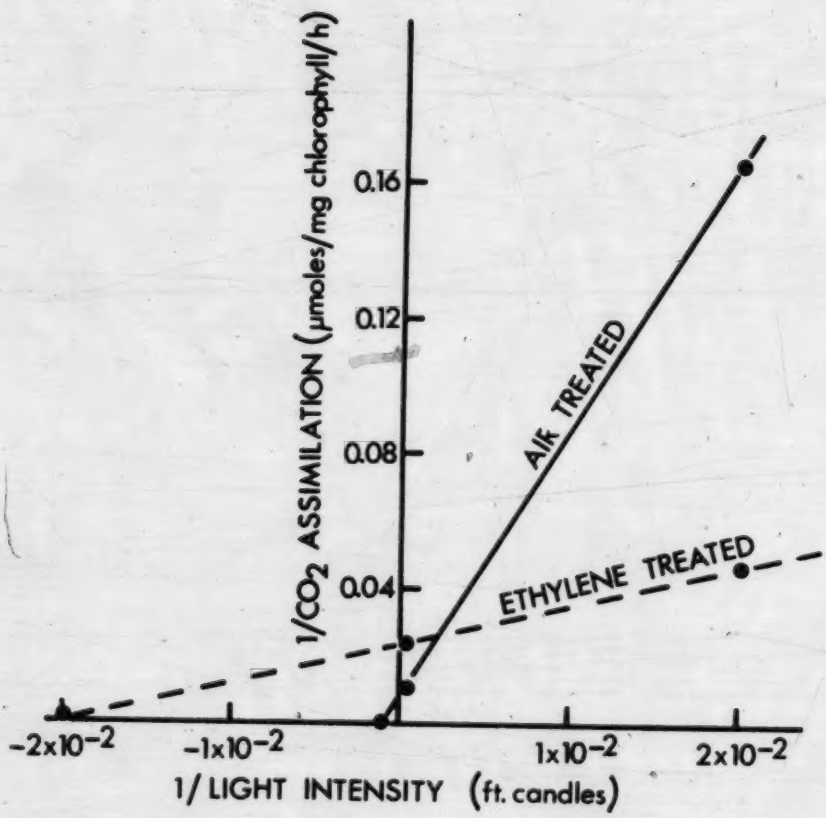


Fig. 24. Double reciprocal plot of light intensity and net CO_2 assimilation by air- and ethylene-treated spinach leaves (after a pretreatment of two days). The results of Fig. 24 were very limited because of only two light intensities, namely 50 ft candles and 2500 ft candles, although the double reciprocal plots of light intensity and net CO_2 assimilation by air- and ethylene-treated spinach leaves for one and three days were indicated a similar trend as that of Day 2 is shown in Fig. 24.

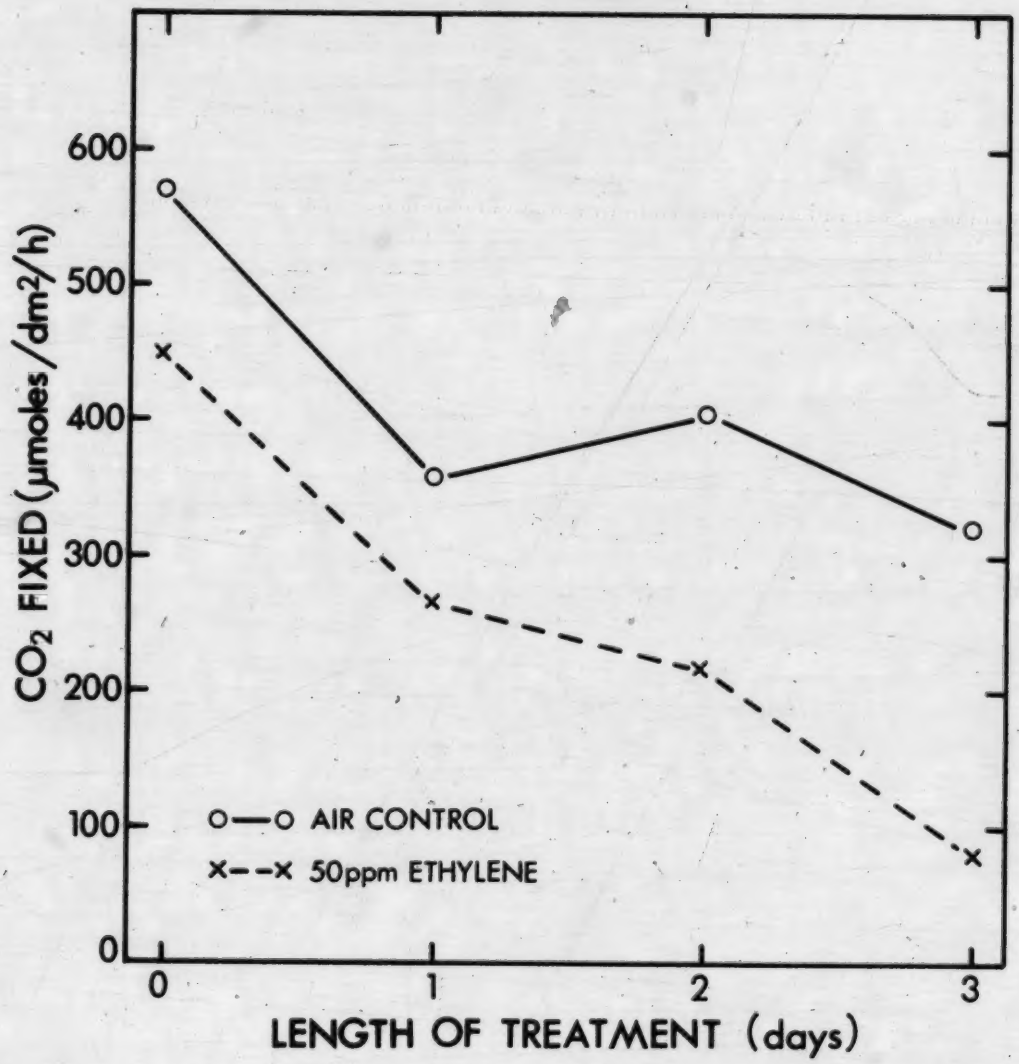


Fig. 25. Net CO_2 assimilation of air- and 50 ppm ethylene-treated spinach leaves at a light intensity of 2500 ft candles. Both kinds of leaves were previously treated in the dark for various lengths of time.

TABLE 17. The effect of 50 ppm ethylene on the respiration* of detached spinach leaves

Time (days)	Air Control			50 ppm ethylene		
	µmoles CO_2 evolved/dm^2/h			µmoles CO_2/dm^2/h		
	Expt. 1	Expt. 2	Average	Expt. 1	Expt. 2	Average
0	33	40	36	56	58	57
1	26	26	26	57	42	50
2	10	22	16	59	30	44
3	28	20	24	81	48	64

*Respiration was measured by the IR CO_2 Analyzer.

4. Effects of ethylene on the ultrastructure of chloroplasts

Two different light intensities were used in the experiment, 50 ft candles and 2500 ft candles.

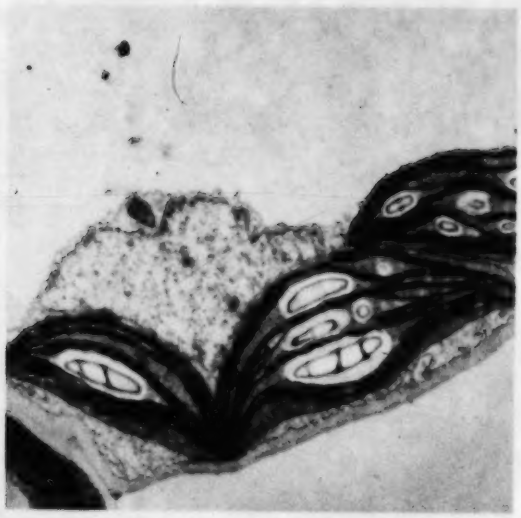
The ultrastructure of chloroplasts was affected by 50 ppm ethylene, and was more pronounced in the effect on chloroplasts when the spinach leaves were exposed to 2500 ft candles as shown in Figs. 26, 27, 28, and 29, than that exposed to 50 ft candles. The untreated samples when fixed immediately after detachment exhibited normal granal and stromal thylakoids and a large number of starch granules were present; very few osmiophilic bodies could be seen. The osmiophilic bodies are believed to be degradative products of thylakoids (Butler and Simons, 1971). These electron-dense bodies increased in number and size with increasing length of ethylene treatment. When spinach leaves were treated with 50 ppm ethylene under 2500 ft candles for three days, the disorganization of the thylakoid membrane systems increased with time (Figs. 26, 27, 28, and 29). The symptoms of swelling or discontinuity of thylakoids, dislocation of grana and sometimes invaginations of the inner membrane of the envelope were observed. The osmiophilic bodies usually occurred within the disorganized thylakoid area or at the end of the thylakoids. These bodies broke the continuity of membrane system. Perhaps the discontinued thylakoids massed together, resulting in the formation of osmiophilic bodies. In spinach leaves treated with 50 ppm ethylene for three days under a light intensity of 2500 ft candles, the outer and inner membranes of chloroplasts were dissolved and osmiophilic bodies and disorganized thylakoids became suspended in the cytoplasm. The disappearance of tonoplast during senescence of plant tissues as

Fig. 26. Effect of 50 ppm ethylene on ultrastructure of spinach leaves at the light intensity of 2500 ft candles for one day. The fifth leaf from the apex of each spinach plant was chosen for the treatment and fixed in glutaraldehyde and osmium tetroxide and stained with uranyl acetate and lead citrate. The electron micrographs show that the chloroplasts of ethylene-treated spinach leaves lose their starch granules and thylakoids start to swell.

(1) fresh leaf (magnification, X 9,500)

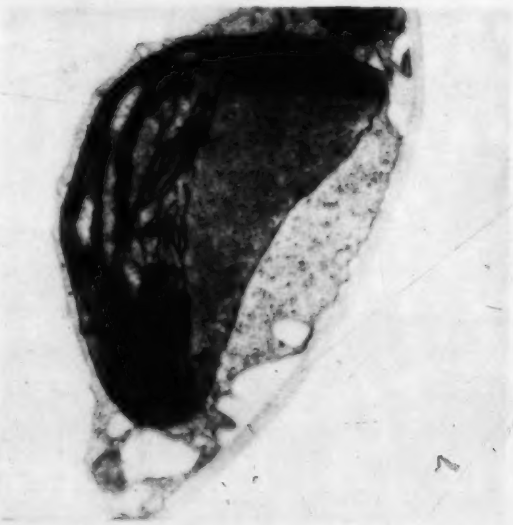
(2) air-treated leaf (magnification, X 9,500)

(3) ethylene-treated leaf (magnification, X 16,250)



a

b



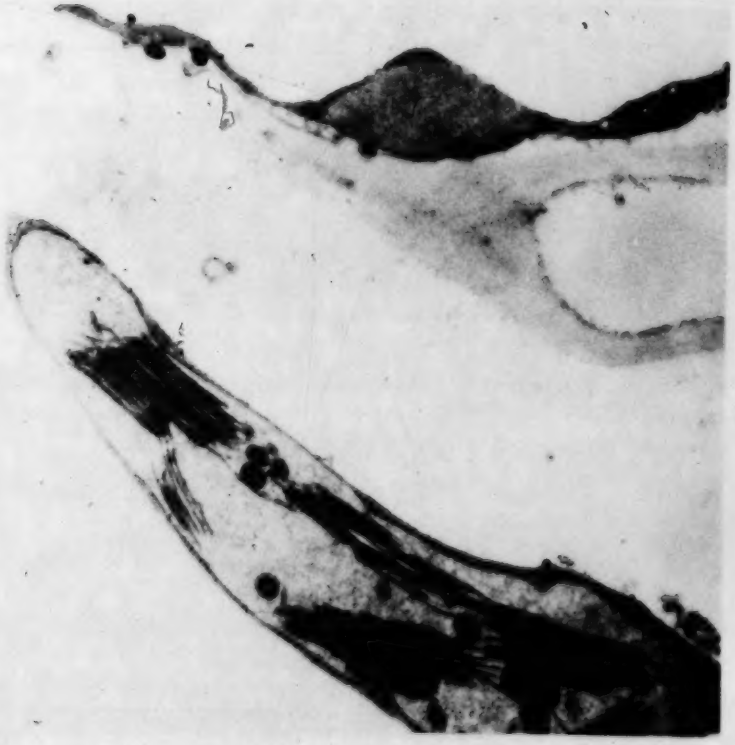
c

Fig. 27. Effect of 50 ppm ethylene on ultrastructure of spinach leaves at the light intensity of 2500 ft candles for two days. Discontinuity of thylakoid start to show out in the second day of ethylene treatment with osmiophilic globules between two grana.

(1) air-treated leaf (magnification, X 21,014)

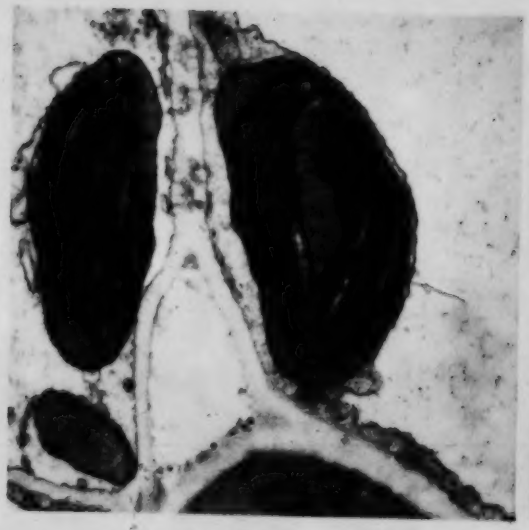
(2) ethylene-treated leaf (magnification, X 21,014)

a



b

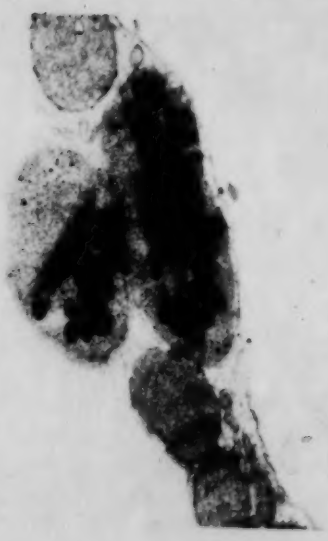
Fig. 28. Effect of 50 ppm ethylene on ultrastructure of spinach leaf at the light intensity of 2500 ft candles for three days. The membrane structure of grana from ethylene treatment is almost totally disrupted whereas air-treated samples still mainatin normal grana structure. Osmiophilic globules increased in number and size because of increase in thylakoid degradation by ethylene treatment. In some cases, the outer and inner membranes of chloroplasts were dissolved (d) and osmiophilic bodies and disorganized thylakoids become suspended in the cytoplasm (b, c). Deposit of globules in the cytoplasm can only be seen in ethylene-treated samples.
(a) air-treated leaf (magnification, X 13,500)
(b, c, d) ethylene-treated leaf (magnification, X 21,250)



a

b

c



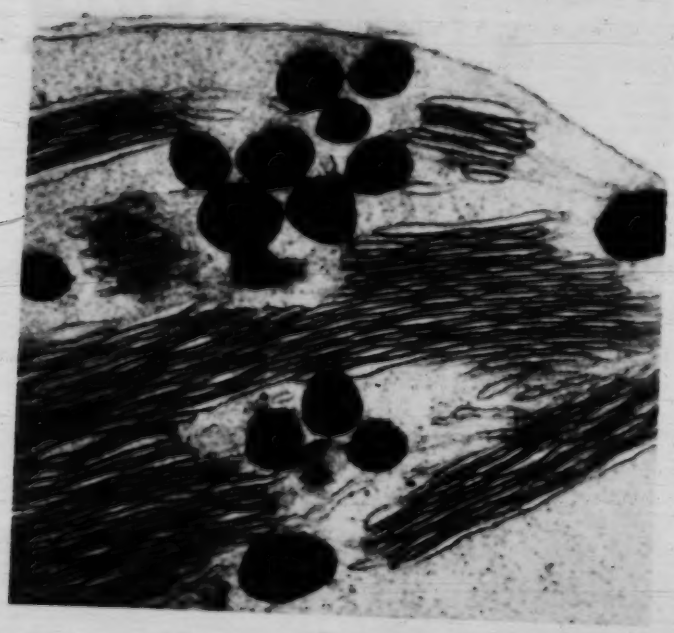
d

Fig. 29. Effect of 50 ppm ethylene on ultrastructure of spinach leaf at the light intensity of 2500 ft candles for three days, high magnification of electron micrograph (both are 62,720 times magnification).

a. air-treated leaf

b. ethylene-treated leaf

a



b

mentioned by Butler and Simon (1971) was not seen in ethylene-treated spinach leaves.

The samples that were treated with air were only slightly affected. No discontinued thylakoids or dissolved chloroplast membranes were observed. Only a few smaller osmiophilic bodies were detected even after three days of exposure (Figs. 28 a and 29 a). The grana of the chloroplasts showed only a slight swelling.

Some starch granules were present in chloroplasts of three day old air controls whereas in ethylene-treated samples, there were no starch granules even after one day of treatment.

When the ethylene treatment was performed under low light intensity (50 ft candles), the ultrastructure of chloroplasts was not markedly affected as compared to that of air controls or after three days of treatment. However, slight differences between air-treated and ethylene-treated samples could be observed even after one day.

DISCUSSION

A. Physiology of the Ethylene-enhanced Chlorophyll Degradation

1. Degree of organization and response to ethylene

Two interesting points have been evidenced in the results. First, *Lemna* cultures were much less sensitive to ethylene than the spinach leaves. Secondly, the unicellular alga *Chlorella* did not apparently respond to ethylene application. It thus appears that the lower the degree of organization of a plant, the lower is its sensitivity to ethylene. It should be pointed out - and this was the reason that *Lemna* and *Chlorella* had been tried - that the two species were intact organisms which were maintained in culture during ethylene treatment while the spinach leaves were detached. On this fact must be the reason for the difference of response of the *Lemna* plants and the spinach leaves. The *Lemna*, being intact and receiving its nutritional elements from the culture medium in a normal way, seems to possess a higher degree of resistance. This fact is related to Burg's finding (1964) that mango fruits which were still attached to the tree did not undergo the ripening process inspite of the very high concentration of ethylene present in its internal atmosphere, while they ripened quickly upon detachment. Burg suggested that an inhibitor of ripening was provided by the plant itself and also proved that the inhibitor was precisely carbon dioxide. Whether these suggestions are right or not, the fact remains that intact plants or plant organs attached to plants are less affected by ethylene than detached organs. Our data thus corroborate Burg's data.

The apparent insensitiveness of *Chlorella* to ethylene is more difficult to explain. Although for practical consideration, further studies of *Chlorella* were not done, especially on its ethylene production, it is interesting to point out that microorganisms generally produce much more ethylene than organisms with higher organization. From work on *Euglena*, Newell (1971) observed that the rate of ethylene production by *Euglena* is as follows: 6,000 µl/kg/h for closed heterotrophic cultures; 160 µl/kg/h for aerated heterotrophic cultures (condition adopted for our *Chlorella* cultures) and 0.2 µl/kg/h for autotrophic cells. Phan (1963) showed that when the ethylene emission of pears was 83 µl/kg/h, the concentration of ethylene in the internal atmosphere was in the range of 100 ppm. This means that all the concentrations applied in this thesis were lower than, or for the most part equal to, the endogenous level of ethylene. Perhaps here lies a possible explanation for the non-effectiveness of ethylene on the *Chlorella* cultures.

2. Efficiency of different ethylene concentrations on chlorophyll degradation

The results (Fig. 6) have shown that the ethylene concentrations of 50 and 100 ppm produced, at long terms, similar effects, and that the concentration of 50 ppm was, therefore, adopted for subsequent studies. Looking at the results from a physiological point of view, it appears that there is a threshold concentration above which the amplitude of the response does not increase. The discrepancies between the responses to 50 ppm and 100 ppm during the first three days could be explained by the fact that the concentration of 100 ppm is markedly higher than the endogenous level of ethylene and, therefore, can produce an immediate effect. The concentration of 50 ppm must be close to the initial

endogenous level, and therefore, was active only when this endogenous level has somewhat decreased. The same type of explanation can be applied to the fluctuating responses to the concentration of 20 ppm. In fact, this concentration must have been for the most part equal and initially lower than the endogenous ethylene level.

3. Interaction of light intensity and ethylene in chlorophyll degradation

Results from the present investigations clearly show that the higher the light intensity, the greater was the detrimental effect of ethylene on chlorophyll. To the author's knowledge, this is a novel finding. There were two linear relationships when the chlorophyll contents were plotted against a logarithmic scale of light intensity. Chlorophyll degradation increased tremendously on changing the light intensity from 500 ft candles to 5000 ft candles.

Photodestruction of chlorophyll is a well known fact. Generally, it is thought to be an oxidation resulting on a protonated chlorophyll radical which can then undergo ring opening, giving rise to an opened chain of tetrapyrrole and a molecule of carbon monoxide (Crespi and Katz, 1972). Ethylene is known to favor oxidative processes, either by inducing oxidative enzymes or by acting as electron acceptor (Spencer, 1969).

Theoretically, when ethylene has accepted two electrons, the resulting product would be ethane (Stahman *et al.*, 1966). Consequently, under this set of conditions, a stimulation of ethane production would be expected. Although no special attention had been given to ethane, the author did notice an increase on the area of the ethane "peak" of gas chromatograms of emanations from spinach leaves kept at 50 ft candles.

The possible biological importance of ethane has been pointed out by Lieberman (1962) who was the first to identify ethane among the "impurities" which were often included in the "ethylene" peak when the gas chromatographic analysis was done at high temperature. Phan (1970) also found that ethylene was always accompanied by three other gaseous compounds, one of which was ethane, the relative proportion of which varied with the age of the tissues and especially Ghooprasert (1971) has demonstrated an increase of ethane production by bean cotyledon mitochondria under stress.

4. Interactions between CO_2 and ethylene

One of the phenomena generally admitted is that CO_2 acts as an antagonist to ethylene. Burg and Burg (1969) even classified it as a competitive inhibitor of ethylene. This idea has been questioned by Phan (1971) who, using Burg's data, calculated that CO_2 must be present at a concentration of at least 200 times that of ethylene to be able to counteract ethylene action. Furthermore, when the ethylene concentration is higher than a certain value, which varies with the tissue studied, its action cannot be reversed by CO_2. The interrelationship between ethylene and CO_2 thus appears to be more complex than suggested by preliminary experiments.

The results obtained in this study with different concentrations of carbon dioxide and 50 ppm of ethylene indicated that the degradation of chlorophyll was not directly proportional to CO_2 concentration. The chlorophyll content of spinach leaves treated with 50 ppm ethylene (without CO_2) was about 30% lower than that of 50 ppm ethylene plus 0.009% CO_2-treated samples. This could be because of the stimulatory effect on the closure of stomata by higher CO_2 concentrations. The

response of stomata adjective closure or opening to higher or lower concentrations of CO_2 is a well known fact (Scarth, 1932; Freudenberger, 1940; Heath, 1940, 1950; Heath and Milthorpe, 1950; Heath and Russel, 1954) in both illuminated as well as darkened leaves and in etiolated leaves (in which chlorophyll could not be detected by fluorescence microscopy) as well as in normal leaves. An increase in CO_2 concentration causes stomata to close and a decrease causes it to open. In 1932, Scarth suggested, and his suggestion was later confirmed by other workers, that the possibility of light-dark responses (light stimulated stomata opening and dark induced stomata closure) of stomata might be entirely caused by photosynthetic removal, and respiratory production, of CO_2 inside the leaf. A light intensity of 50 ft candles, which is the intensity used for most of the experiments in this thesis, may not be high enough to have the CO_2 uptake exceed that evolved by respiration. In fact, when CO_2 assimilation by detached spinach leaves was studied, the trace line on the recorder went up indicating a CO_2 evolution higher than uptake. Furthermore, ethylene stimulated respiration of spinach leaves (Table 17, p. 112). Consequently, it can be inferred that the concentration of CO_2 in the intra- and intercellular spaces was in fact higher than 0.009%, which was the concentration of CO_2 applied exogenously during the treatment.

The results obtained with the CO_2 concentration higher than 1% showed that there is a toxic effect in addition to the effect of ethylene. The mechanism of the combined effect of CO_2 and ethylene on the chlorophyll contents of spinach leaves is still unknown. More work needs to be done to understand how different CO_2 concentrations affect intracellular pH, ultrastructure of chloroplast and chloroplast

physiology and metabolism. When CO_2 concentration increases, there is a greater tendency of the following reaction to move to the right:

$$CO_2 + H_2O \longleftrightarrow HCO_3^- + H^+$$

$$CO_2 + H_2O + HCO_3^- + H^+ \longleftrightarrow 2H_2CO_3$$

H_2CO_3 then dissociates to form $H^+ + HCO_3^-$. The pKa of this reaction is 3.77, which is quite low for a leaf cell. However, in the leaf cell the situation might not be as simple as a single chemical reaction. Cho (1966) proposed that the pheophytinization of chlorophyll a was more sensitive to acid than chlorophyll b, probably because of the structural difference in the attachment at C_3; a greater positive charge on the nitrogen atoms of chlorophyll b makes them less sensitive to attack by proton. If this proposal is true, the ratio of Chl a/Chl b should decrease in CO_2-treated spinach leaves. However, this was not the case. The ratio of Chl a/Chl b in both air-treated and ethylene-treated spinach leaves was similar in the sealed jar (higher CO_2) (Table 4, p. 68) to that in a continuous flow (lower CO_2) (Table 1, p. 55). CO_2 might also intervene by forming a complex called "chlorophyll bicarbonate" by Willstatter and Stoll (quoted by Rabinowitch, 1945) which could be the first step in the conversion of chlorophyll into pheophytin, through the formation of $MgCO_3$. The concentration of 10% CO_2 must have a special biological significance as Malhotra and Spencer (1974) have shown that the stimulation of the activity of the "membrane bound" (but not the "highly purified") Mg^{++}-dependent adenosine triphosphatase (ATPase) by ethylene (100 ppm) and in the presence of CO_2 was rapid from 1% to 5% CO_2 and then began to decline at 10% CO_2. From the fact that only the "membrane bound" enzyme was affected, we could infer that in the present investigation the degradation of chlorophyll implies a prior

alteration of the thylakoid membranes. It is also possible that there is a similar effect of ethylene and CO_2 on chloroplast ATPase.

The results relative to high concentrations (25-50%) of CO_2 must be considered cautiously because of a number of factors. First, high CO_2 concentrations cause the wilting of leaves. According to Chichester and McFeeters (1971) the dessication of the leaves was one of the ways to protect the chlorophyll in leaf crops during storage. Therefore, the wilting of spinach leaves could protect chlorophyll against degradation. Secondly, high CO_2 concentrations may induce pH changes, accumulation of intermediate metabolites of respiration and of photosynthesis. Other metabolic alterations could also take place.

B. Biochemical Aspects of Chlorophyll Degradation as Affected by Ethylene

Although the work covered only chlorophyll degradation, the general problem of senescence also needs some consideration. For leaves, chlorophyll destruction can be viewed as a component of senescence. Furthermore, ethylene is known as a senescence promoting agent.

1. Effects of ethylene on the protein content of detached spinach leaves

In the present study, treatment with 50 ppm ethylene resulted in an increase in the soluble proteins of spinach leaves during the first day of treatment at 50 ft candles and then a decrease after the second day to a value lower than that of the fresh leaves (Day 0), whereas in the air-treated leaves, the soluble proteins remained constant with only a slight increase during the first six hours. These results indicated that ethylene might stimulate the protein synthesis during the first

day of treatment. This may have been a result of increased synthesis of catabolic enzymes, *e.g.* chlorophyllase, protease, cellulase, etc. (Abeles, 1973). Ethylene has been shown not only to increase cellulase synthesis, but also to regulate the movement of cellulase from the cytoplasm to the cell wall through the membrane (Abeles and Leather, 1971).

Proteases are responsible for the rapid decrease in protein content of senescent tissues. Several investigators have shown that Fraction 1 protein was the main protein component lost in senescing tobacco (Dorner *et al.*, 1957; Kawashima *et al.*, 1967a,b; Kawashima and Mitake, 1969) and perilla (Kannangara and Woolhouse, 1968) and during the senescence of detached barley leaves (Peterson and Huffaker, 1975). Because most of the total soluble proteins lost in barley leaves was accounted for by a decrease in ribulose 1,5-diphosphate carboxylase protein, Peterson and Huffaker (1975) proposed that the chloroplast was a major site of degradation in early senescence. They also found that cycloheximide essentially prevented the loss of chlorophyll, ribulose 1,5-diphosphate carboxylase protein and its activity, and completely inhibited the increase in proteolytic activity against azocasein. Since chloramphenicol had little effect on these changes, it was suggested that the proteolytic activity was developed in the cytoplasmic 80 S ribosomes. This was also found in the present study. The protein content of spinach leaves increased within the first day and consequently decreased below the original (0 time) level after one day of ethylene treatment. Furthermore, three days after the application of cycloheximide to spinach leaves, there was a complete protection of chlorophyll against ethylene, whereas with chloramphenicol, there was

only a slight effect (Table 5). Thus it appears that the effects of ethylene on the degradation of chlorophyll in spinach leaves is mediated via protein synthesis (probably) in the cytoplasm.

2. Effect of ethylene on chlorophyllase activity

Chlorophyllase activity increased during the bleaching of spinach leaves treated with 50 ppm ethylene (Fig. 9). The activity was retarded by the addition of 1.2% CO_2 in the ethylene stream (Fig. 11) or by treatment in sealed jars where the accumulation of CO_2 occurred. In both cases, less chlorophyll was degraded. These results indicate that there is a probable involvement of chlorophyllase in the degradation of chlorophyll during ethylene treatment. This involvement could be a direct stimulation of chlorophyllase action on dephytylation of chlorophyll and subsequent disorganization of the chloroplast or an indirect effect of ethylene on the degradation of chlorophyll as an event during ethylene-enhanced senescence of spinach leaves.

3. Effects of ethylene on Chl a/Chl b ratio and the degradation products of chlorophyll

Because of the similarity in the ratio of Chl a/Chl b between air- and ethylene-treated leaves, the effects of ethylene on the degradation of chlorophylls a and b can be considered to be equal. This would suggest that the mechanism of ethylene action was not mediated through the removal of Mg^{++} because if Mg^{++} was removed and chlorophylls degraded, the ratio of chlorophyll a to b should be changed according to Cho (1966) who proposed that in the pheophytinization of chlorophyll (removal of Mg^{++}) by acid, chlorophyll a is more sensitive than chlorophyll b.

The degradation of chlorophyll *in vivo*, as effected by ethylene

appears to proceed further than dephytylation or pheophytinization as no large derived compounds, *e.g.* chlorophyllides, pheophytins or pheophorbides were observed. This was also found in senescent leaves (Seyhold, 1943) and in the biodegradation of chlorophyll in a mixotrophic mutant of *Chlorella pyrenoidosa* (Ziegler and Schanderl, 1969). It has been reported that the particles of Sephadex LH20 in chloroform show a specific affinity towards compounds having a -COOH group (Pharmacia Handbook, 1966). Among the porphyrin compounds that are commonly observed in plant extracts, those that have a -COOH group are the chlorophyllides and pheophorbides. These were not found (except in trace) in air- and ethylene-treated samples. This is an indication that if chlorophyllase activity is increased upon ethylene treatment, the action of ethylene does enhance also further degradation of the products of chlorophyllase action.

If we now look at the degradation products, apart from a slight qualitative difference in band 1 (after Sephadex LH20 gel chromatography) between these two samples (ethylene-treated samples exhibited maxima of absorption in chloroform at 682, 462 and 407 nm, whereas air-treated samples had maxima at 676, 498 and 408 nm) only quantitative differences could be detected between air- and ethylene-treated samples. this means that ethylene enhances only the degradative processes rather than new pigment biosynthesis; this finding corroborates what has already been shown on other effects of ethylene, in fruit physiology for instance, Biale (1960) clearly demonstrated that ethylene, when applied exogenously only accelerates the onset of the climacteric rise but does not alter its overall chracteristics. Another interesting point deserves to be pointed out. In ethylene-treated spinach leaves, not

only chlorophylls but all pigments namely, carotenes and the other carotenoids also decreased. The symptom of yellowing of ethylene-treated leaves thus appears to be a mere unmasking of the yellow color by decreasing the green pigmentation rather than increasing the carotenoid content. As chlorophylls and carotenoids are essential for normal granal structure and photosynthetic activity in green plants, their lower contents would result in disorganized thylakoid membrane structure and subsequent reduced photosynthetic activity.

4. Effects of Cytokinins

In Tables 8 and 9, cytokinins appeared to act as antagonists against ethylene on its effects on chlorophyll degradation. It is interesting to note that kinetin was more effective than N-6-benzyl-adenine (NBA). These compounds are only different in N^6-substitution, one contains furfuryl group (kinetin), another contains benzyl group (NBA). Kinetin is probably more soluble than NBA. Some reports in the literature have stated that cytokinins, including kinetin and NBA, are not easily translocated. In order to check this possibility, disks of spinach leaves were placed on the surface of cytokinin solutions. The effectiveness of this procedure was rather slight (Table 8 a). One reason for this rather small improvement could be because of the large amount of ethylene produced by the disks through the wounding effect. Cytokinins usually cause a two- to four-fold increase in ethylene production (Abeles, 1973). Plants showing a positive response include bean (Abeles *et al.*, 1967), blueberries (Forsyth and Hall, 1968), radish (Radin and Loomis, 1969), sorghum (Owens *et al.*, 1971) and pea (Burg and Burg, 1968; Fuchs and Lieberman, 1968). The concentration required for a maximum effect was 10^{-6}M, and threshold effects were

seen at 10^{-8}M (Fuchs and Lieberman, 1968). Ethylene production by spinach leaves treated with 9.3×10^{-6}M (2 µg/ml) of kinetin was not as high as by other plant species reported in the literature. In fact, when the spinach leaves were dipped in a kinetin solution and the ethylene produced was immediately collected and measured, the production of ethylene was first inhibited by 50% for leaf disks and 75% for whole leaves. Only after one hour did the production of ethylene increase, and thereafter it continued to increase up to day 3 and remained three times higher than that of air control. A similar trend was observed in NBA-treated leaves. The stimulation of ethylene production by cytokinins may be explained on the basis that the endogenous indole acetic acid (IAA) is preserved by cytokinins (Lau and Yung, 1974) through the suppression of increase of IAA oxidase during senescence because the rate of ethylene production parrallels the internal free IAA level (Abeles, 1972). Furthermore, Lau and Yang (1973) provided evidence indicating that cytokinins suppressed the conversion of IAA to IAAsp which was inactive in inducing ethylene production by IAA tracer studies.

The "protection" of chlorophyll against ethylene action might be caused by the involvement of cytokinins in the process of regeneration of membranes and reorganization of thylakoid membranes. Indeed, cytokinin treatments initiated regeneration of membranes and reorganization of grana in chloroplasts of senescent tobacco leaves (Svestinikova *et al.*, 1966) and were necessary for the organization of stroma thylakoids into grana of the chloroplasts of growing tobacco callus cells (Laetsch and Stetler, 1967). Some studies indicate that cytokinin affects leaf senescence by slowing the breakdown of protein (Kuraishi, 1968; Mizrahi *et al.*, 1970; Shibaoka and Thimann, 1970; Skoog and

Armstrong, 1970; Tavares and Kende, 1970) coupled with degradation of chlorophyll rather than by stimulating protein synthesis (Skoog and Armstrong, 1970).

C. Effects of Ethylene on the Photosynthetic Apparatus and Activity

1. Effects of ethylene on chloroplast ultrastructure

As already pointed out in the Results section, the most striking effect of ethylene on the ultrastructure of the chloroplasts is the rupture of the intergranal lamellae. The grana are consequently suspended in the stroma, and generally coalesce to form large stacks. The micrograph of chloroplasts of a Day 3 ethylene-treated leaf shows that the stacked thylakoids are also swollen and eventually rupture. It can, therefore, be inferred that the integral lamellae are cut first because they are more exposed than those enclosed in the thick structure of the grana, but that ethylene does affect all membranes in the same way.

The possible significance of the enhanced activity of chlorophyllase for the structure of the thylakoid has already been exposed. The influence of light intensity on the chlorophyll content has also been examined. It should be pointed out here that the marked structural alteration reported occurred at high light intensity. At 50 ft candles, there was little difference as compared to the structure of the chloroplasts extracted from leaves kept in air. Cran and Possingham (1974) also found that disks from mature spinach leaves under high light intensity exhibited loss of chloroplast structure within four days, while in darkness, much of the thylakoid system persisted for as long as seven days.

Osmiophillic globules could be seen in chloroplasts from both

air- and ethylene-treated leaves. However, their number was much higher and the size of globules much larger in the ethylene-treated ones. It is generally agreed that osmiophillic globules are mainly lipophillic in nature (although compounds of other natures could give the same electron-dense image), and are derived from the degradation of thylakoid membranes. In this connection, it should be remembered that ethylene has a high affinity for lipids and is believed to attack preferentially the lipid components of bio-membranes.

It is interesting to note that the staining of the starch granules varied with the length of ethylene treatment. In untreated or shortly treated chloroplasts, the starch granules appeared white on the electron micrographs. Then their image darkened. In the 3-day treated samples, only the shape of the starch granules remained. The interpretation for these changes is that as the chloroplasts lose their activity and respiration is enhanced (a well known effect of ethylene), the starch is hydrolyzed and the darkened images of starch granules are indicative of shorter chains of glucans, probably resulting from the hydrolysis of the starch chains, and are finally used up, hence the hollow spaces between lamellae.

2. Effects of ethylene on Hill reaction

Ethylene did not appear to have any effect on the Hill reaction of "chloroplasts" isolated from spinach leaves before, or after one day of treatment with ethylene. However, after two days, in the ethylene-treated samples, the grana of chloroplasts were found to break down and separate from each other. This might produce an effect on the photo-system II. As expected, the activity of the Hill reaction was decreased after two days of ethylene treatment.

From the above investigation, the inhibition of the Hill reaction of "chloroplasts" isolated from spinach leaves after two to three days of treatment with 50 ppm ethylene could be explained on the basis of the effect of ethylene on chloroplast membrane systems, which in turn could induce the loss of enzymes and electron carriers of photosystem II.

3. Carbon dioxide fixation by isolated chloroplasts

The chloroplasts isolated from spinach leaves obtained from plants grown in growth chambers or purchased from Edmonton City Market during winter months were inactive when CO_2 was supplied as Na_2CO_3. Therefore, the effect of ethylene on $^{14}CO_2$ fixation by isolated chloroplasts could not be measured by incubating isolated chloroplasts and $Na^{14}CO_3$ in an oxygen monitor. This might be because of a lack of carbonic anhydrase activity in the spinach grown in a growth chamber or because of leakage of this enzyme during the isolation of chloroplasts (Gibbs, 1966). The CO_2 fixation by isolated chloroplasts is also more sensitive to shocks than O_2 evolution. Possible rupture of chloroplasts during isolation could lead to a loss of the enzymes required for CO_2 fixation, such as ribose-1,5-diphosphate carboxylase (RuDP carboxylase). Graham and Reed (1971) reported that the carbonic anhydrase might be more important in CO_2 transport. Firstly, this enzyme may be associated with the RuDP carboxylase and may increase CO_2 fixation by increasing the available free CO_2 at the active site or by increasing the affinity of RuDP carboxylase for CO_2. Secondly, carbonic anhydrase may overcome the permeability barrier for CO_2 at the chloroplast membrane thereby increasing the rate of CO_2 transfer to ribulose diphosphate carboxylase in the chloroplast. Thirdly, carbonic anhydrase by virtue of

the reaction(s) it catalyzes a) may rapidly make available the large proton gradient through the thylakoid membrane, which gradient is probably associated with photophosphorylation; b) may be involved in a rapid buffering action regulation, for example, the proton gradient and HCO_3^- concentration and the phosphorylative and ion exchange capacities of the chloroplasts. The carbonic anhydrase catalyzed the interconversion of CO_2 (which is the species fixed by ribulose-1,5-diphosphate carboxylase) and HCO_3^- or CO_3^{--}. The carbonic anhydrase could also be important in regulating the rates of carboxylation and also in the regulation of the Hill reaction and photophosphorylation, both of which require or are stimulated by CO_2 species on the acid side of their pH optima.

The other possibility is that spinach grown in the growth chamber or purchased from the Edmonton City Market (originally grown in California) during winter could contain chloroplasts in which the envelope membrane of the "chloroplast" might be more fragile or porous. As a result, during the isolation, NADP could leak out, as it did in isolated chloroplasts from spinach leaves when plants were grown at the University of Alberta farm, aged at 0°C for five hours (Fig. 18), in which the activity of O_2 evolution could be restored by the addition of 0.5 mM NADP. This result indicated that perhaps the envelope membrane of isolated chloroplasts was damaged during storage. In Jensen and Bassham's report (1968) Herber and Santarius concluded from their studies with non-aqueous isolated chloroplasts that the chloroplast membrane *in vivo* was impermeable to NADPH and NADP. This suggests that if the envelope membrane was intact there should normally be no leaking out of NADP.

4. Effects of ethylene on the net CO_2 assimilation by spinach leaves

The data on the effect of net CO_2 assimilation by spinach leaves under low (50 ft candles) and high (2500 ft candles) light intensities are quite interesting in that they provide some new insight into the effect of ethylene. The data presented in Fig. 21 show that under high light intensity, ethylene gradually inhibited the net CO_2 fixation. Although the rate of CO_2 fixation by air-treated samples also decreased within the first day and then kept constant throughout the experiment, yet the rate after three days of exposure was almost three times that of ethylene-treated samples. On the other hand, the data in Fig. 23 show that under low light intensity, after an initial decline, the net CO_2 assimilation of ethylene-treated spinach leaves increased steadily. However, the rate of CO_2 assimilation after two days was still lower than that under high light intensity (compared to the results present in Fig.21). Also the rate of CO_2 fixation by air-treated leaves was much lower under low light intensity than that under high light intensity.

All these observations taken together point to the fact that the leaves, after three days of exposure to ethylene, exhibit similar rates of CO_2 fixation under either low or high light intensity. This behavior probably is the result of ethylene-induced changes in the chloroplast ultrastructure (see Figs. 26-29). Perhaps once the ultrastructure was disrupted, the effect of different intensities of light on the CO_2 fixation was not evident. In order to explain the increase in CO_2 fixation by ethylene-treated leaves under low light intensity, one needs to consider other probable phenomena where CO_2 may be evolved.

It is to be expected that plant tissues would evolve CO_2 as a result of the so-called dark respiration and of photorespiration. As the author is not aware of any report indicating the effect of light on dark respiration, it is logical to speculate that ethylene might have inhibited the photorespiration under the two light conditions. If we assume that photorespiration was reduced in ethylene-treated leaves, then the net amounts of CO_2 fixed under low light intensity must be greater, despite the fact that ethylene caused chloroplast ultrastructure disruption, than that of air-treated ones. On the other hand, under high light intensity the net CO_2 assimilation by air-treated samples would be greater than that by ethylene-treated samples because of ethylene-induced disruption of chloroplast ultrastructure.

Another explanation for such a differential effect of ethylene on the net CO_2 assimilation at low and high light intensities could be the ethylene-induced promotion of light saturation for photosynthesis by spinach leaves. It is obvious from Fig. 24 that the light intensity for half of maximum activity of photosynthesis for ethylene-treated spinach leaves was decreased by a factor of approximately 20 compared to that of air-treated leaves. It means that the light saturation point for photosynthesis of ethylene-treated spinach leaves is about twenty times lower than that of air-treated samples.

The net CO_2 assimilation at 2500 ft candles by spinach leaves previously treated with 50 ppm ethylene in the dark was affected less compared to those treated with the same concentration of ethylene but in the presence of light (50 ft candles) (Fig. 25). This might be because of a lesser effect of ethylene on chloroplast structure in the darkness than in the light (50 ft candles).

The Hill reaction at 2500 ft candles of chloroplasts isolated from spinach leaves previously treated with ethylene at 50 ft candles for two days was inhibited by 40% compared to the air-treated sample, whereas the net CO_2 fixation at 2500 ft candles by ethylene-treated leaves similarly treated previously with ethylene was inhibited by 60% compared to that by air-treated samples. This lack of an exact correlation between the effects of ethylene on CO_2 fixation and the Hill reaction could be explained by the fact that ethylene might affect some other center besides photosystem II.

Goldney and Van Steveninck (1972) reported that RuDP carboxylase (ribulose diphosphate carboxylase EC 4.1.1.39) activity was relatively unaffected by exogenous ethylene (25 ppm) during ten days of treatment of detached *Nymphoides indica* leaves. If this was also true for detached spinach leaves, the ethylene could affect either photosystem I or photophosphorylation, or others. Furthermore, they also found that 25 ppm ethylene inhibited both malic enzyme (L-malate:NADP oxidoreductase EC 1.1.1.40) and 6-p-gluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase EC 1.1.1.44) compared to air controls, *i.e.* ethylene inhibited the decarboxylation of malate and 6-p-gluconate. The inhibition of these decarboxylating enzymes should lead to an increase in the net CO_2 assimilation. However, observations of decrease in net CO_2 assimilation of ethylene-treated spinach leaves were made to the contrary which should indicate that the effects of ethylene on *Nymphoides indica* and spinach leaves are different. In fruits, the phenomenon of the climacteric rise in respiration corresponds well with the increase in CO_2 evolution which may result mainly from the decarboxylation of malate (Rhodes *et al.*, 1969). They reported that 300-500

ppm exogenous ethylene exerted a slight effect on the level of decarboxylative activity of malic enzyme. However, ethylene eliminated the lag phase in the decarboxylation involved in ripening apples. The system in ripening apples may also be different from that in detached spinach leaves. The net CO_2 assimilation of ethylene-treated spinach leaves was lower than that of the air control.

CONCLUSION

It has been long known that ethylene enhances the degreening of plant tissues. Considerable research had been done towards the application of ethylene for the industrial purpose of fruit ripening. Fewer have been the researchers who did look into this phenomenon from a physiological and biochemical point of view, especially as to its consequence for the photosynthetic ability of the treated tissues. This work was an attempt to fill some parts of that gap.

The degreening, *i.e.* the disappearance of the green coloration of plant tissues, seems to be the result of a number, if not a sequence, of degradation steps. First, ethylene has been found to enhance the activity of the enzyme chlorophyllase, the action of which is the detachment of the porphyrin "head" from the phytol "tail" of the molecule of chlorophyll. If one accepts the schematic structure of chloroplast membrane proposed by Anderson (1975a) this first step already causes a disruption of the thylakoid membrane. This point will be reexamined later. The detachment of the porphyrin part, named chlorophyllide, would not yet cause the degreening because chlorophyllide has an absorption spectrum quite similar to that of chlorophyll; in fact, chlorophyllide is the chromatophore of chlorophyll. Furthermore, in the analysis of the degradation products, chlorophyllide was not found and neither was pheophytin nor pheophorbide, which rules out the degradation of porphyrin ring as a primary step. Further degradation of the chlorophyllide must, therefore, take place very rapidly. The biochemical process of this degradation is indicated by the synergistic action of

ethylene and light. It must be an oxidation process similar, if not identical, to the photooxidation reaction, which is at the same time the first step of photosensitization of chlorophyll in the photosynthetic sequence and the starting point for the rupture of the tetrapyrrole ring. According to several workers, especially Crespi and Katz (1972), this reaction results in the formation of a linear tetrapyrrole and carbon monoxide. This aspect has not been explored in this work and deserves further investigation. On the other hand, the discovery of the possible interference of light on the ethylene effect is an essential guide for subsequent investigations in this field. As complete darkness is also harmful to chlorophyll and chloroplast structure, an adequate compromise must be found; we have adopted a low light intensity (50 ft candles) in our experiments.

The increase in chlorophyllase activity was found to correlate initially with the increase in soluble protein content of ethylene-treated leaves. Both cycloheximide and chloramphenicol proved to be efficient in inhibiting the ethylene-induced chlorophyll degradation, however, only cycloheximide exerted a complete inhibition. This is indicative of the fact that the enzymes responsible for the degradation of chlorophyll are synthesized *de novo* in the cytoplasm. If this is actually the case, then these enzymes must be transported rapidly through the chloroplast double envelope into the stroma. Here also, ethylene must have an important role, as it has been shown to decrease the "organization resistance of cells and tubular organelles" (Bain and Mercer, 1964). However, this increased transport through the chloroplast envelope appears to be dissociated from the structural damage on the envelope, as ruptured chloroplast envelope could only be seen after

three days of ethylene treatment. Once the degradative enzymes have passed through the envelope, they must remain dissolved in the stroma because the first part of the chlorophyll bearing system that are attacked are the stroma (or intergranal) lamellae.

If we accept the idea of the primary hydrolysis of ester bond between phytol and chlorophyllide in the thylakoid membrane structure through the action of chlorophyllase, then the results concerning the effect of ethylene on the photosynthetic activity, especially on CO_2 fixation, become clear. Indeed, when the photosynthetic efficiency is calculated on the milligram chlorophyll basis, the trend was similar to that calculated on a leaf area basis. This means that not all the pigments, the estimation of which is based on their absorption values at 649 and 665 nm (Vernon, 1960), and which is commonly called "chlorophyll", are photosynthetically active. The part that is active represents the intact chlorophyll molecules which are structurally implanted in the thylakoid membrane up to the time of extraction, the other part represented by products resulting from chlorophyllase action, *i.e.* chlorophyllides, does increase the "chlorophyll" weight but is not apt to support photosynthesis. In other words, the degradation of chlorophyll affects directly the integrity of the lamellar structure of the thylakoid and consequently the photosynthetic viability of the chloroplasts, and only indirectly the degreening process, which results from other enzymic or non-enzymic degradative reactions.

Perhaps it would be interesting to place the degradation of chlorophyll in the larger context of ageing and senescence. Chlorophyll disappearance is indeed a part of the senescence process in leaves. Moreover, ethylene is known to be a senescing agent. Therefore, within

the context of this work, the results obtained are related to leaf senescence. However, it should be remembered that chlorophyll degradation per se is not always correlated with senescence.

A last point which deserves special attention is the interaction between ethylene and CO_2. The theoretical aspect of this interaction has already been examined in the Discussion. From the practical point of view, two ranges of CO_2 concentration (very low or higher than 10%) are interesting as far as chlorophyll preservation is concerned. In the very low concentration range, the "protective" effect of CO_2 seems to be quite powerful. In fact most of the "controlled atmospheres" contain 1 to 5 percent CO_2 and it was indeed observed that chlorophyll contents of plant tissues were higher in "controlled atmospheres" than in air. Higher concentrations of CO_2 would not be advisable because of the wilting, and also the risks of the onset of anaerobic processes in the tissue.

This work is by no means exhaustive and leaves some unexplained points: (1) the exact role of CO_2 below, at and above the concentration of 10%, (2) the interrelation between ethylene and light, (3) whether or not ethylene causes a lowering of the light saturation value for photosynthetic CO_2 assimilation, (4) ethylene effect on purified chlorophyllase. Further ultrastructural investigations especially by means of the freeze-etching technique should also be done in order to detect fine alterations induced by ethylene.

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